Universidade de Aveiro

Departamento de Biologia

2016

Bruno Miguel Castanheira Prates Campino

Diversidade de espécies de *Botryosphaeriaceae* associadas a plantas na região de Rostov (Rússia)

Diversity of *Botryosphaeriaceae* species on plants from the Rostov region (Russia)

DECLARAÇÃO

Declaro que este relatório é integralmente da minha autoria, estando devidamente referenciadas as fontes e obras consultadas, bem como identificadas de modo claro as citações dessas obras. Não contém, por isso, qualquer tipo de plágio quer de textos publicados, qualquer que seja o meio dessa publicação, incluindo meios eletrónicos, quer de trabalhos académicos.

Universidade de Aveiro

Departamento de Biologia

2016

Bruno Miguel Castanheira Prates Campino

Diversidade de espécies de *Botryosphaeriaceae* associadas a plantas na região de Rostov (Rússia)

Diversity of *Botryosphaeriaceae* species on plants from the Rostov region (Russia)

Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Aplicada, realizada sob a orientação científica do Doutor Artur Jorge da Costa Peixoto Alves, Investigador Principal do Departamento de Biologia da Universidade de Aveiro

Dedico este trabalho aos meus pais, sem os quais nada disto teria sido possível.

o júri

presidente

Prof. Doutor João António de Almeida Serôdio
professor auxiliar com agregação do Departamento de Biologia da Universidade de Aveiro

Doutora Liliana Tavares dos Santos
Investigadora em pós-doutoramento do Centro de Estudos do Ambiente e do Mar da Universidade de Aveiro

Doutor Artur Jorge da Costa Peixoto Alves
investigador principal do Departamento de Biologia da Universidade de Aveiro

agradecimentos

Ao meu orientador, Doutor Artur Jorge da Costa Peixoto Alves, pela inestimável oportunidade que me deu para poder entrar no mundo da Microbiologia e da Biologia Molecular, por ter sempre a segurança e tranquilidade, humanidade e paixão pelo que faz que me motivaram a dar inicio a este trabalho e que foram cruciais para que a conseguisse levar até ao fim.
À Doutora Carla Barradas, por me ter recebido de forma tão maravilhosa e por me ter acompanhado e ajudado na difícil fase inicial de ambientação, assim como todo a orientação dada durante o restante trabalho.
A todo o pessoal do Microlab, foi um prazer e uma aprendizagem acompanhada de muitos momentos e conversas inesquecíveis.
Aos meus pais que possibilitaram tudo isto, desde a formação às lições de vida, que até ao dia de hoje definem quem sou.
Ao meu irmão que tem sido sempre uma fonte de inspiração e que me motiva para ser sempre uma pessoa melhor hoje, do que a pessoa que era ontem.
A todos os meus amigos com os quais partilhei alegrias, tristezas, emoções fortes aliadas às mais diversas fases que a vida pode proporcionar.
Um grande obrigado a todos pois esta vida não seria a minha sem todos vós.
Obrigado por estarem presentes durante a minha viagem.

palavras-chave

Fungos, Plantas Hospedeiras, Rostov, Diversidade, *Diplodia*, *Dothiorella*, *Phaeobotryon*,Tipagem.

resumo

Este estudo teve como objectivo avaliar a diversidade de espécies da família *Botryosphaeriaceae,* usando uma abordagem multifacetada combinando dados morfológicos e moleculares, associadas a uma grande e diversa coleção de amostras de plantas hospedeiras provenientes da região de Rostov na Rússia. Os fungos isolados foram inicialmente analisados por reação em cadeia de polimerase usando primer BOX para avaliar a diversidade genética global dos isolados. Isolados representativos de cada grupo foram selecionados para posterior identificação filogenética combinando duas regiões de ADN, usando o espaçador interno transcrito (ITS) em conjunto com o factor de elongamento de transcrição 1-alfa (EF1-α). Os resultados revelaram três géneros, *Diplodia*, *Dothiorella* e *Phaeobotryon. Dothiorella* foi o género mais representado e prevalente de toda a coleção e a espécie *Dothiorella sarmentorum* claramente mais dominante. Duas potenciais novas espécies foram identificadas neste trabalho, uma espécie pertencente ao género *Dothiorella* e uma outra ao género *Phaeobotryon*. Várias espécies identificadas foram relatadas pela primeira vez na Rússia e em vários hospedeiros diferentes.

keywords Fungi, Plant hosts, Rostov, Diversity, *Diplodia*, *Dothiorella*, *Phaeobotryon*, Fingerprinting**.**

abstract This study aimed to assess the diversity of *Botryosphaeriaceae* species associated with a large and diverse collection of plant hosts from the Rostov region in Russia, using a polyphasic approach combining morphological and molecular data. Fungal isolates obtained were initially subjected to a fingerprinting by BOX-PCR in order to evaluate the overall genetic diversity of the collection. Selected isolates representative of each group were further identified by a phylogenetic approach combining two gene regions, the internal transcribed spacer (ITS) of the ribosomal DNA cluster and part of the translation elongation factor 1-alpha (EF1-α). The results revealed three genera, namely *Diplodia, Dothiorella* and *Phaeobotryon*. *Dothiorella* was the most represented and prevalent genus throughout the samples studied and the species *Dothiorella samentorum* was clearly dominant. Two putative new species were identified in this work, one from the genus *Dothiorella* and another from *Phaeobotryon*. Several of the species are reported from Russia for the first time a large number of new host associations were identified.

Index

Figure List

Table list

1. - Introduction

1.1 - The family *Botryosphaeriaceae*

The order *Botryosphaeriales* comprises 7 families, *Aplosporellaceae*, *Botryosphaeriaceae*, *Melanopsaceae*, *Phyllostictaceae, Planistromellaceae*, *Saccharataceae* and *Septorioideaceae.* Of these, *Botryosphaeriaceae* is the largest and more diverse, including 22 genera, (Crous et al., 2015; Phillips et al., 2013; Slippers et al., 2013; Wyka and Broders, 2016). The species in this family are commonly known for being endophytes, latent or opportunistic pathogens, necrotrophic or saprobic mainly on woody hosts, having a worldwide distribution and also a very wide range of hosts. Some species are important pathogens known for affecting several hosts in several countries and continents (Slippers and Wingfield, 2007). Diseases caused by these fungi are almost exclusively associated with stress conditions exerted on the hosts (Phillips et al., 2013; Schoeneweiss, 1981; Slippers and Wingfield, 2007; Smith et al., 1994).

Species in this family can infect the host plants via wounds in the tissue and also infect via stromata and other openings in healthy tissue (Arx and Müller, 1954; Brown, II, 1981; Kim et al., 1999; Michailides, 1991; Slippers and Wingfield, 2007; Smith et al., 1994). When the infection is yet to be well established in the host, hyphae from *B. dothidea* produce glyoxyssomes, these structures have been studied by (Kim et al., 1999, 2001, 2004; Slippers and Wingfield, 2007) and it is suggested that in case of nutrient deficiency and/or stacked by the host defence mechanisms these structures allow the fungi to survive. Still the majority of new infections happen through horizontal transmission, via conidia (asexual spores) (Smith et al., 2001).

When these fungi are able to infect a host, the infection remains in its endophytic phase if the host is healthy and not under stress. These infections have been reported in all plant tissues, xylem of stems, branches, leaves, flowers, fruits, seed capsules and seeds (Cilliers et al., 1995; Johnson et al., 1992; Kim et al., 2004, 2001; Lupo et al., 2001; Slippers and Wingfield, 2007; Smith et al., 1996). These fungi cause disease only when the host is under stress conditions (Blodgett et al., 1997; Ma et al., 2001; Paoletti et al., 2001; Schoeneweiss, 1981;

Slippers and Wingfield, 2007; Slippers et al., 2013; Smith et al., 1994; Stanosz et al., 2002).

In terms of which stress factors causes disease expression, the general idea is that any stress factor with enough influence to affect the host physiology can trigger the disease expression. Some factors are likely to have higher impacts, like drought, extensive physical damage, pathogens such as insects and parasites, snow, frosty weather, plant competition, characteristics of the soil and climate of the area (Blodgett et al., 1997; Ma et al., 2001; Paoletti et al., 2001; Schoeneweiss, 1981; Slippers and Wingfield, 2007; Smith et al., 1994; Stanosz et al., 2002).

When the disease starts to manifest, a range of symptoms associated with *Botryosphaeriacea*e develops. These include twig, branch, root and stem cankers, die-back of leaders, shoots and branches, seed capsule abortion, collar rot, dumping of seedlings, blue stain decline and ultimately death of the host (Ahumada, 2003; Bega et al., 1978; Brown and Britton, 1986; Gure et al., 2005; Johnson et al., 1992; Lupo et al., 2001; Mohali et al., 2007; Sánchez et al., 2003; Slippers and Wingfield, 2007; Smith et al., 1994; Swart and Wingfield, 1991).

These fungi have been found in almost every plant tissue from healthy plants, so it is believed that almost all species of *Botryosphariaceae* have an endophytic phase. The term endophytic is due to the presence of these fungi inside healthy plant tissue (Saikkonen et al., 1998; Slippers and Wingfield, 2007; Stone and Petrini, 1997). Normally most infections have little biomass inside the host, too small to take any toll on the hosting organism, usually entering in a latent phase and there it stays until stress upon the host occurs (Slippers and Wingfield, 2007; Smith et al., 2001). Endophytes may have a potential to become mutualists by playing a protective role against parasitic agents (Carroll, 1988, 1990; Saikkonen et al., 1998; Sieber, 2007; Slippers and Wingfield, 2007). Mutualist roles for *Botryosphaeriaceae* species are not yet verified, still some situations strongly suggest this possibility. In case of parasitic insects the loss of the injured and infected tissue can impair the development and progression of the aggressor by poisoning or removing the food supplies (Carroll, 1990; Slippers and Wingfield,

2007). In case of senescence of leafs and branches that are no longer efficient in photosynthesis, will save the host resources (Carroll 1988; Slippers & Wingfield 2007).

These fungi have great potential to cause diseases and plant losses, due to the proximity within plants the prevalence of the infections are usually higher. Some reported cases illustrates very well the capability to cause destruction and severe impairment in agriculture sector in terms of production and the quality of the products. *Diplodia sapinea* is responsible for major losses on pine plantations in South Africa, up to 55% after hail damage and die-back, being the most economic important pathogen of pine trees in this country and in many other parts of the world, this pathogen is responsible for big losses also in USA, New Zealand and in Europe (Zwolinski et al. 1990; Reay et al. 2006; Wet et al. 2000; Smith et al. 2001; Blodgett et al. 1997; Stanosz et al. 2002; Luchi et al. 2005; Maresi et al. 2002; Slippers & Wingfield 2007).

Species of *Botryosphaeriaceae* with wide range of hosts may have more potential to cause damaging disease expression. In terms of new infections, in new hosts this can possibly represent an advantage, meaning that probably the host has never developed any resistance to the fungus infection. *Diplodia sapinea* and *Diplodia seriata* are good examples to support this claim, but some other species such as *Dothiorella sarmentorum* also have a wide host range and geographical distribution but has no major losses reported due to this species (Ahumada 2003; Wet et al. 2000; Pavlic et al. 2007; Niekerk et al. 2004; Slippers & Wingfield 2007; Parker & Gilbert 2004; Slippers 2005).

The fundamental challenge that *Botryosphaeriaceae* represents, is the need to create and apply quarantine measures to endophytic organisms that are most likely to not develop any disease and symptoms unless the host is subjected to stress conditions. This means, that plants, seeds and fruits travel all around the globe without manifesting any symptoms and when the disease starts to express, it is already too late to take any preventive control measures, it is usually only then that the infection is detected.

These problems will probably be aggravated by climate change, which can induce longer and more frequent periods of stress. Acute weather, for example, either drought or floods, will possibly originate more cases of massive disease expressions due to opportunistic infections.

Combining the absence of any doable and efficient measures of quarantine and aggravated weather conditions due to climate change represents a much possible scenario with major implications worldwide (Desprez-Loustau et al., 2006; Slippers and Wingfield, 2007).

1.2 - Identification and description tools

Identification of *Botryosphaeriaceae* species is essentially based in the shape, size and disposition of certain structures of the asexual morph type, such as, conidiomata, conidiophores, conidiogenous cells and conidia (Denman et al., 2000; 2013, 2008). A brief description of the structures used for identification and description of species is given below.

Conidiomata are the reproductive structures (fruiting bodies) of the asexual morph type of *Botryosphaeriaceae* that can be seen in infected tissue. These structures have variable morphology having either thin or thick walls, arranged in uniloculate or multiloculate pycnidial, it develops in a preformed stroma, there for called stromatic.

Conidiophores are the cells that produce conidia through the abstriction of the base, these are usually quite rare and its presence differs even in isolates of the same species. If there are any, normally they are hyaline with a smooth surface, have thin walls and its shape is more or less cylindrical.

Conidiogenous cells, that later give origin to conidia, are hyaline, have a smooth surface and thin walls, shaped between longiform to ampuliform. Conidia initially grow holoblastically at the end of the conidiogenous cells, then the following conidia are formed either through internal proliferation of the tissue resulting in periclinal thickenings, or growing percurrently originating annelations.

Both kinds of growing can be seen at the same time even in the same conidiogenous cell.

Paraphyses are sterile hyphal cells intertwined with the conidiogenous tissue, sometimes reaching above the forming conidia, the presence or absence of these structures can be used for species identification but it is a character that can be or not even in the same species.

Conidia are the spores of the asexual morph type of *Botryosphaeriaceae*. Two major types of conidia morphology can be found in this family, in one the conidia have thin walls and are smaller, the other one have thick walls and are larger. The first type, *Fusicoccum*-like, conidia are normally fusiform, ovoid or ellipsoid, hyaline and aseptate at first. In some cases they may become pale brown, septate and with thicker walls when mature. The second type, *Diplodia*-like, conidia are hyaline or brown, have thick walls and can become septated and are shaped in ovoid form. The surface is usually smooth but some are striated, inside the spores sometimes a verruculose texture can be seen (Phillips et al., 2013).

Description of species of *Botryosphaeriaceae* in the past were based on general morphological species concepts used for other fungi families, however this approach resulted in a underestimation of the true diversity in this family (Taylor et al., 2000). Species concept for *Botryosphaeriaceae* was initially based in the general morphology of ascospores, ascomata and stromata (sexual morph stage). Considering that the sexual morph type is very rare and harder to grow, compared to the diversity, abundance, easy to isolate, to grow and to induce sporulation provided from the asexual morph stage. Using the asexual morph added more resolution and enabled the description of many more species (Denman et al., 2000). The characters of the asexual morph stage used for species descriptions and identification are conidiomata, conidiogenous cells, conidiophores, paraphyses and conidia the last is described in size, shape, colour, septation and L/W ratio. Despite all this, intraspecific variations are very high even between isolates of the same species, this implied that the morphological species concept was not adequate or not enough to truly resolve all the species in the *Botryosphaeriaceae* complex.

The advances in DNA sequence data allowed new tools to be used for distinguishing species, differences in DNA sequences was combined with morphological characteristics by (Phillips et al. 2005; Crous et al. 2006; Smith et al. 2001; Alves et al. 2004; Alves et al. 2007) with huge success. Using the 28S rRNA gene (Crous et al., 2006) was able to separate 10 lineages within the family correlating to different morphological characteristics, still a large clade remained unresolved corresponding to *Diplodia*, *Lasiodiplodia*, and other pigmented conidia.

Molecular methods used amplified ribosomal DNA restriction analysis as fingerprinting method to rapidly and efficiently identify the isolates to the species level, this method also allows to study many isolates at once (Alves et al., 2007). The ITS loci is widely used to phylogenetically distinguish species of fungi but lacks the needed resolution to separate many cryptic *Botryosphaeriaceae* species. The combination of two different gene data sets such as ITS and EF1-α improved immensely the ability to resolve some groups of cryptic species, yet some groups were to close phylogenetically to be separated properly (Slippers et al., 2004).

A new order was proposed based on multigene phylogenies, SSU, LSU, EF1-α and RNA polymerase II gene (RPB2) sequences, *Botryosphaeriales*, to solely accommodate the family *Botryosphaeriaceae* (Schoch et al., 2006). The *Diplodia/Lasiodiplodia* complex was separated using ITS, ribosomal RNA Large Subunit (LSU), EF1-α and β-tubulin sequence data (Phillips et al., 2008).

Many groups cannot be distinguished only by morphological analysis, for example, *Dothiorella* and *Spencermartinsia*, differ only in the apiculi of the ascospores. This means that phylogenetic data cannot be completely supported by morphological data to distinguish different species and even different genera. In order to not underestimate true species diversity, a multi-locus phylogenetic approach combining ribosomal RNA Small Subunit (SSU), LSU, ITS, EF1-α and β-tubulin enables a separation of the genera (Phillips et al., 2013; Slippers et al., 2013). These results can be supported at some length by the differences in morphology of the genera, especially for conidia. (2013, 2008; Slippers et al., 2013).

2. - Objectives

The Rostov region (Russia) is located in the southern end of the Eastern Europe between the Black Sea and the Caspian Sea. The region has a continental climate and most of the territory is farmland, only near 6% of the region's area is covered by woods and bushes. Very little is known about the mycobiota inhabiting the plants from this region especially regarding the fungal family *Botryosphaeriaceae*.

This study results from collaboration with a researcher from Rostov region in Russia. It was undertaken with the overall aim to assess the diversity of *Botryosphaeriaceae* species associated with a large collection of diverse plant hosts from the Rostov region. For this, a polyphasic approach combining morphological and molecular data was used.

3.- Materials and Methods

3.1 - Fungal Isolation

Fungi were isolated from 41 samples of plant hosts collected in the Rostov region between the years of 2013 and 2014 (Table 1). Isolations were made directly from ascomata or conidiomata. These were cut through vertically with a sterile scalpel, placed in a drop of sterile water and then spread over the surface of a plate of ½ strength potato-dextrose agar (½ PDA; Merck, Germany). The plates were incubated at room temperature (≈25ºC) overnight and single germinating spores were transferred to new ½PDA plates in order to establish single spore cultures from all samples.

Table 1 – List of samples from which all isolates were obtained. (ms) – multiple spore cultures.

Index	*Host plants*	Isolates
P-001	*Crataegus crus-gallii* L.	P1-2B
		P1-2C
		P1-2D
P-002	*Crataegus crus-gallii* L.	P2-2A
		P2-2B
P-003	*Sorbus aucuparia* L.	P3-2A
		P3-2B
P-004	*Euomymus europaeus* L.	P4-2A
		P4-2B
P-005	*Prunus armeniaca* L.	P5A
		P5B
P-006	*Sorbus intermedia* (Ehrh.) Pers.	P6A
		P6B
P-007	*Cornus sanguinea* L.	P7A
		P7B
P-008	*Tamarix ramosissima* Ledeb.	P8A
		P8B
		P8?
		P8ms
P-009	*Menispermum canadense* L.	P9A
		P9B
		P9C
		P9D

P-010	*Juglans regia* L.	P10-2A
		P10-2B
P-011	*Juglans regia* L.	P11-2A
		P11-2B
P-012	*Cornus sanguinea* L.	P12A
P-013	*Cornus sanguinea* L.	P13B
P-014	*Syringa vulgaris* L.	P14A
		P14B
		P14-2A
		P14-2B
P-015	*Rosa cf. centifolia* L.	P15-2A ms
		P15-2B ms
P-017	*Fraxinus pennsylvanica* Marshall	P17-2 A
P-018	*Ligustrum vulgare* L.	P18-2
P-019	*Celastrus orbiculatus* Thunb.	P19A
		P19B
P-025	*Forsythia × intermedia*	P25B
P-028	*Cornus sanguinea* L.	P28-2
		P28-2ms
P-034	*Stephanandra incisa* Thunb.	P34A
		P34B
P-035	*Acer negundo* L.	P35-2ms
P-037	*Acer negundo* L.	P37 ms
		P37-2A
		P37-2B
		P37-2C
P-039	*Fraxinus pennsylvanica* Marshall	P39 ms
		P39-2A
		P39-2B
		P39-2C
P-040	*Styphnolobium japonicum* L.	P40A
		P40B
P-046	*Lycium barbarum* L.	P46A
		P46B
P-050	*Cornus sanguinea* L.	P50A
		P50B
P-064	*Fraxinus pennsylvanica* Marshall	P64A
		P64B
P-065	*Acer tataricum* L.	P65A
		P65B
P-067	*Cornus sanguinea* L.	P67-1
		P67-2
P-069	*Morus alba* L.	P69A
		P69B

P-075	*Forsythia* × *intermedia*	P75-1
		P75-2
P-076	*Prunus fruticosa* L.	P76-1
		P76-2
P-078	*Sorbus aucuparia* L.	P78
P-079	*Cotoneaster sp.*	P79-1
		P79-2
P-081	*Fraxinus pennsylvanica* Marshall	P81
P-083	*Rhamnus cathartica* L.	P83
P-085	*Pyrus communis* L.	P85
P-087	*Acer tataricum* L.	P87
P-092	*Cornus sanguinea* L.	P92-1
P-094	*Acer negundo* L.	P94

3.2 - Morphological Characterization

For the isolates of interest, sporulation was induced by growing them on (¼ strength PDA) or 2 % water agar containing double autoclaved pine needles. Plates were incubated at room temperature (≈25ºC) with diffused day light for 3 to 4 weeks until pycnidia could be seen on the pine needles and/or on the surface of the medium. These were then dissected and mounted in 100% lactic acid for microscopic observation. Observations of micromorphological characteristics (e.g. conidial size, shape, colour, striation, septation, conidiogenous cells, presence of paraphyses) of the isolates were made with a Nikon 80i microscope and digital images were recorded with a Nikon DS-Ri1 camera. Measurements were made with the Nikon Nis-Elements imaging software (Nikon, Japan). A minimum of 100 conidia were measured for each isolate and mean, standard deviation and 95% intervals were calculated.

The growth rates studies were performed for the temperatures of 5, 20, 25, 30 and 35 ºC. For culturing, 90mm Ø PDA plates, were inoculated at the center using a 5mm plug and left to incubate for 5 days. After the 5 days, growth was measured with a ruler.

3.3 - Molecular Characterization

3.3.1 - DNA extraction

Genomic DNA was extracted from fungal cultures grown on (½PDA) plates for 7 days at room temperature (≈25ºC). Mycelium growing on the plates was scraped to a 2 ml microtube with 500µl of 100 mM Tris, pH 8.0, 10 mM EDTA, 2% SDS (TES) buffer, everything was mixed well and then the mixture was heated at 100 ºC and for 3 minutes. Afterwards the tubes were placed in an ice bath for 10 minutes and 10 µl of proteinase K at the concentration of 10 mg/ml was added followed by incubation at 65 ºC for 30 minutes during which occasional mixing by agitation or inversion to improve the digestion was done. Salt concentration was increased by adding 140 µl of sodium chloride at 5 M, followed the addition of 65 µl of 10% cetyltrimethylammoniumbromide (CTAB) solution. All tubes were mixed and incubated at 65 ºC for another 30 minutes, once again swirling occasionally to improve the reactions. After incubation 1 ml of chloroform:isoamylalcohol in the proportions of 24:1 respectively was added to the tubes and carefully mixed by inversion, followed by a 30 minutes incubation on ice. Mixture was then centrifuged for 10 minutes at 12000 rpm at 4 ºC, supernatant (±800 µl) was transferred to a 1.5 ml microtube followed by the addition of 225 µl of ammonium acetate (NH_4OAc) at 5 M concentration and mixed carefully by inversion, tubes were then placed in ice for another 30 minutes incubation. Tubes were again centrifuged for 10 minutes at 12000 rpm and 4 ºC and the supernatant (1000 µl) was transferred to a new 1.5 ml microtube in which 500 µl of ice-cold isopropanol was added and mixed carefully before incubating on ice for 30 minutes. A last centrifugation was performed for 10 minutes at 12000 rpm and 4 ºC and supernatant was discarded. The pellet was dissolved in 50 µl of 100 mM Tris, pH 8.0, 10 mM EDTA (TE) buffer and stored at -20 ºC until further use. This protocol was adapted from (Möller et al., 1992).

3.3.2 - BOX-PCR fingerprinting

For BOX-PCR mixtures, each reaction tube contained 6,25 µl of NZYTaq 2x Green Master Mix (NZYTech, Lisboa, Portugal), 15,75 µl of sterile HPLC-grade water, 2µl of primer BOXA1R at the concentrarion of 10 pmol/ml and 1µl of template DNA, total volume of final reaction mixture was 25 µl. Negative controls contained sterile water instead of the template DNA.

PCR conditions are described in (table 2) all procedures according to. PCR amplicons were separated using a 1,5% agarose gel with Tris-acetate-EDTA (TAE) 1X buffer at 80 V for 2h45m. A Thermo Scientific™ sm0331 DNA Ladder Mix was loaded in both sides of the gel. The gels were then stained with ethidium bromide in order to visualize the amplifications using a UV transilluminator. Gels were scanned using a GelDoc XR+ system (BioRad) and saved as TIFF file to be inserted and analysed with GelCompar II software (Applied Maths). The gels were uploaded after a database was created, then normalised making use of the DNA Ladder Mix standards, the background subtraction was toggled on and any bands detected by the software were revised and corrections were made when necessary. During the analysis the intensity of the bands were not taken into account, similarity levels between bands profiles were computed using the Pearson coefficient. Cluster analysis was performed by unweighted pair group method using arithmetic averages (UPGMA). Using the dendrogram obtained with the BOX-fingerprinting (Figure.1) analysis representatives of each clade were selected for identification by DNA sequencing.

Table 2 – PCR conditions

Cycles	PCR	Initial denaturation	Denaturation	Annealing	Extension	Final Extension
30	BOX	95 ºC - 5 min	94 ºC - 1 min	53 ºC - 1 min	65 ºC - 8 min	65 ºC - 16min
35	ITS	95 ºC - 5 min	94 ºC - 30 s	50 ºC - 30s	72 ºC - 1 min30s	72 ºC - 10min
30	EF1-α	95 ºC - 5 min	95 ºC - 30 s	52 ºC - 30s	73 ºC - 45s	73 ºC - 10min

3.3.3 - Sequencing and phylogenetic analyses

Identification of the isolates was performed by amplification and sequencing of the ITS region of the rDNA and the EF-1α using the primer pairs ITS1 (White et al., 1990)/NL4 (O'Donnell, 1993) and EF1-688F/EF1-1251R (Alves et al., 2008), respectively.

For PCR mixtures, each reaction tube contained 6,25 µl of NZYTaq 2x Green Master Mix (NZYTech, Lisboa, Portugal), 15,75 µl of sterile HPLC-grade water, 2µl of primer (1µl forward and 1µl reverse) at the concentration of 10 pmol/ml and 1µl of template DNA, total volume of final reaction mixture was 25 µl. Negative controls took sterile water instead of the template. DNA. PCR conditions are described in (Table 2) all procedures according to (Alves et al., 2004). PCR amplification was assessed and fragments separated using a 1,5% agaroses gel with TAE 1X buffer ran in the same buffer at 80 V for 1h10m, a Thermo Scientific™. DNA Ladder Mix ran in both sides of the gel. The gels were then stained with ethidium bromide in order to visualize the amplifications using GelDoc XR+ digital imaging system (BioRad).

The amplified PCR fragments were purified with the DNA Clean and Concentrator™-5 kit (Zymo Research, California, USA) and sequenced at GATC Biotech (Germany). The nucleotide sequences were read and edited with FinchTV 1.4.0 (Geospiza Inc http://www.geospiza.com/finchtv).

All sequences were checked and aligned with ClustalX v. 1.83 (Thompson, 1997), using as pairwise alignment parameters (gap opening = 10, gap extension = 0.1) and multiple alignment parameters (gap opening = 10, gap extension = 0.2, transition weight = 0.5, delay divergent sequences = 25 %). Alignments were manually checked and adjusted when needed with Bioedit (Hall, 1999). Phylogenetic analyses of sequence data were done using MEGA7 (Kumar et al., 2016) for Maximum-Likelihood (ML) analysis. The best fitting DNA evolution model to be used in ML analysis was calculated, node reliability was determined with 1000 bootstrap replications and the generated trees were rooted to an outgroup.

To validate the identification and the final analysis other sequences of already described species in published works were retrieved from GenBank, a total of 179 sequences, combining two DNA regions.

4. - Results

A total 80 isolates belonging to *Botryosphaeriaceae* family were obtained from 25 different plant species. In this study the initial approach focused on BOX-PCR fingerprinting to rapidly analyse the overall genetic diversity within all isolates (Figure 1). The 80 isolates were divided in to 6 main clusters potentially representing species. Several representatives of each cluster were chosen for further identification by ITS and EF1-α- sequencing. A total of 31 isolates were selected.

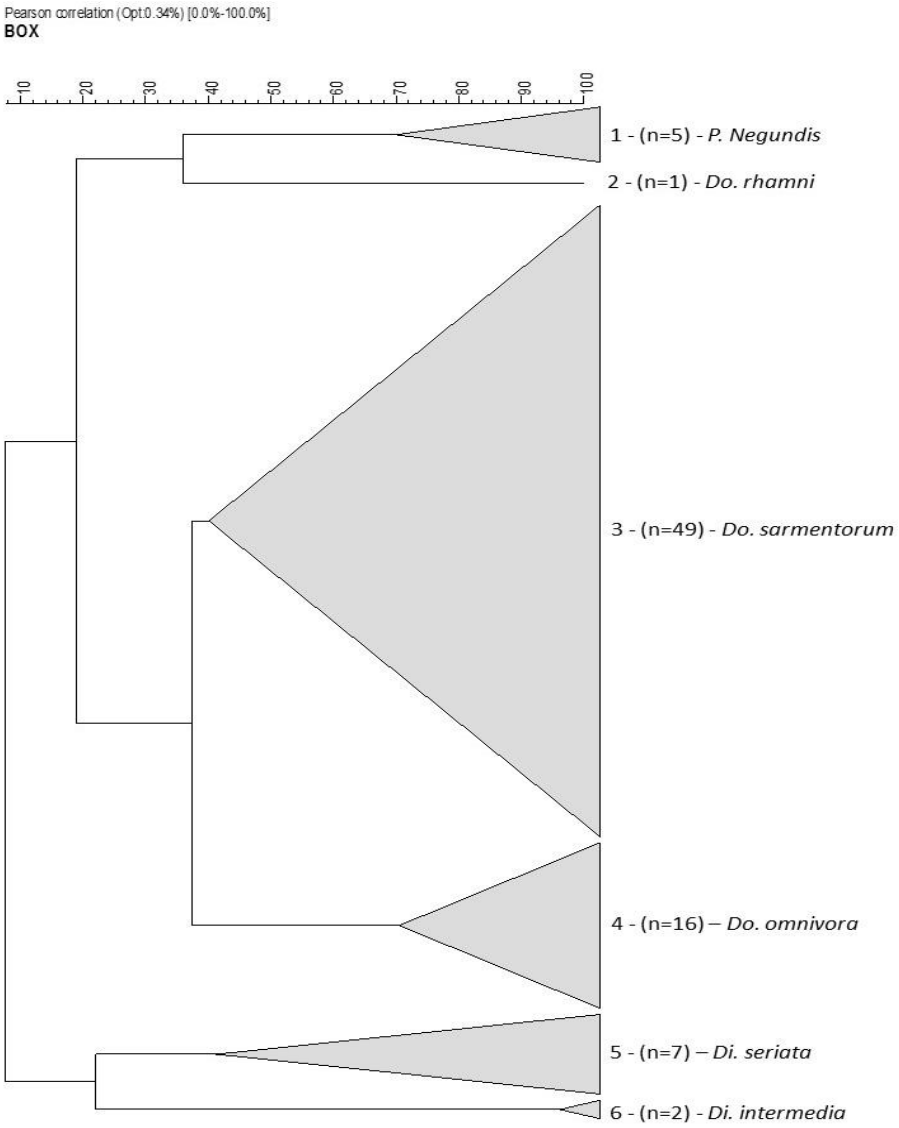

Figure 1- UPGMA cluster analysis based on the Pearson coefficient of BOXPCR fingerprints obtained with primers BOXA1R. Similarity is indicated as a percentage. Species clusters are numbered from 1 to 6 and the number of isolates per cluster is given

All 80 isolates revealed 6 different *Botryosphaeriaceae* species that were isolated from 25 different host species. To assess the host ranges of the species a search was done using the SMML Fungus-Host Distribution Database (Farr and Rossman, 2016).

After an initial analysis using BLAST search of ITS loci, three genera were found and the analysis of the dataset was done separately for each one, namely *Diplodia*, *Dothiorella* and *Phaeobotryon*.

4.1 - *Diplodia* ML tree (Figure 2)

The ML tree shows a total of 19 well separated clusters and was rooted to *Spencermartinsia viticola* as outgroup. All known species of *Diplodia* were used in the alignment and the isolates clustered with two different species. Isolate P5B grouped with *Diplodia intermedia* with high bootstrap value (82%), while isolates P8ms and P14-2B grouped with *Diplodia* seriata with high bootstrap values (87%).

Di. seriata was found on *Menispermum canadense* L. and *Di. intermedia* was isolated from *Sorbus aucuparia* L. and *Sorbus intermedia* (Ehrh.) Pers. (Table 4).

4.2 - *Dothiorella* ML tree (Figure 3)

The ML tree shows all sequenced isolates and the ones retrieved rooted *S. viticola*, and all known species of *Dothiorella* that matched with the retrieved sequences, except for 1 unmatched clade, with low values of bootstrap, strongly suggesting that it is a new species in the genera, described in (Li et al., 2016) and also in this work. The 26 isolates grouped with in 3 clusters corresponding to 3 different species, 17 isolates grouped with *Dothiorella sarmentorum*, 4-2B, 46B, 1-2B, 11-2B, 76-2, 40B, 40A, 81, 79-2, 76-1, 37-2B, 69A, 50B, 12A, 6B and 7B. 8 isolates grouped with *Dothiorella omnivora* 39ms, 9A, 28-2, 28-2ms, 39-2A, 78, 94 and 67-1. Isolate 83 initially did not group with any known species, but it was recently described as a new species, *Dothiorella rhamni* (Li et al., 2016).

Dothiorella sarmentorum was found on *Acer negundo* L., *Acer tataricum* L., *Celastrus orbiculatus* Thunb., *Cotoneaster* sp., *Cornus sanguinea* L,. *Fraxinus pennsylvanica* Marshall, *Juglans regia* L., *Lycium barbarum* L., *Parthenocissus quinquefolia* L. Planch., *Prunus fruticosa* Pall., *Tamarix ramosissima* Ledeb., *Salix babylonica* L., *Sorbus aucuparia* L., *Styphnolobium japonicum* L. Schott and *Syringa vulgaris* L.. *Dothiorella omnivora* is reported in *Acer negundo* L., *Cornus sanguinea* L., *Cotoneaster laxiflorus* J. Jacq. ex Lindl., *Fraxinus pennsylvanica* Marshal and *Sorbus aucuparia* L. (Table 4).

A new species of *Dothiorella* represented 1% (n=1), *Dothiorella rhamni*, the given name takes its origin from the host which it was isolated from *Rhamnus cathartica* L. (Li et al., 2016).

4.3 - *Phaeobotryon* ML tree *(Figure 4)*

The ML tree shows 5 clusters one as outgroup, *S. viticola* and 4 ingroup corresponding to all species of the genera known to date. The isolates did not group with any know species described in published works, clearly suggesting that these represent a new species within the genus *Phaeobotryon*. This species was recently described in (Daranagama et al., 2016) and also described further in this work. The clusters had high bootstrap values between each isolate of each clade suggesting a strong and reliable analysis.

Table 3 – Isolates used in this work and isolates used for DNA comparison with information about species, host, origin, collector and gene sequences reference codes.

Species	Isolate number	Host	Origin	Collector	Sequences	
					ITS	EF-1α
Di. africana	CBS120835	*Prunus persica* (L.) Batsch	South Africa	U. Damm	EF445343	EF445382
	CBS121104	*Prunus persica* (L.) Batsch	South Africa	U. Damm	EF445344	EF445383
Di. agrifolia	CBS132777	*Quercus agrifolia* Née	USA	S. Lynch & A. Eskalen	JN693507	JQ517317
	CBS132778	*Quercus agrifolia* Née	USA	S. Lynch & A. Eskalen	JQ411413	JQ411444
Di. alatafructa	CBS124933	*Pterocarpus angolensis* DC	South Africa	J. W. M. Mehl & J. Roux	FJ888461	FJ888446
	CBS124931	*Pterocarpus angolensis* DC	South Africa	J. W. M. Mehl & J. Roux	FJ888460	FJ888444
Di. allocellula	CBS130408	*Acacia karroo* Hayne	South Africa	J. Jam & M. Gryzenhout	JQ239397	JQ239384
	CBS130410	*Acacia karroo* Hayne	South Africa	J. Jam & M. Gryzenhout	JQ239399	JQ239386
Di. bulgarica	CBS124254	*Malus sylvestris* (L.) Mill.	Bulgaria	S. G. Bobev	GQ923852	GQ923821
	CBS124135	*Malus sylvestris* (L.) Mill.	Bulgaria	S. G. Bobev	GQ923852	GQ923820
Di. corticola	CBS112549	*Quercus sube* L.	Portugal	A. Alves	AY259100	AY573227
	CBS112546	*Quercus ilex* L.	Spain	M. E. Sánchez & A. Trapero	AY259090	EU673310
Di. cupressi	CBS168.87	*Cupressus sempervirens* L.	Israel	Z. Solel	DQ458893	DQ458878
	CBS261.85	*Cupressus sempervirens* L.	Israel	Z. Solel	DQ458894	DQ458879
Di. fraxini	CBS124133	*Lonicera niger* L.	Spain	J. Luque	GQ923856	GQ923824
	CBS124131	*Fraxinus ornus* L.	Italy	S. G. Bobev	GQ923855	GQ923823
Di. intermedia	P2-2B	*Sorbus aucuparia* L.	Russia	Timur Bulgakov	This work	This work
	P5B	*Sorbus intermedia* (Ehrh.) Pers	Russia	Timur Bulgakov	This work	This work
	CBS124462	*Malus sylvestris* (L.) Mill	Portugal	A.J.L Phillips	GQ923858	GQ923826
	CBS112556	*Pyrus communis* L.	Portugal	A.J.L Phillips	AY259096	GQ923851
Di. malorum	CBS124129	*Malus sylvestris* (L.) Mill	Portugal	A.J.L Phillips	AY259095	GQ923827
	CBS124130	*Malus sylvestris* (L.) Mill	Portugal	A.J.L. Phillips	GQ923865	GQ923833
Di. mutila	CBS230.30	*Phoenix dactylifera* L.	USA	L.L. Huillier	DQ458886	DQ458869

Species	Isolate number	Host	Origin	Collector	Sequences	
					ITS	EF-1α
	CBS112554	*Pyrus communis* L	Portugal	A.J.L Phillips	AY259095	DQ458870
Di. neojuniperi	CPC22753	*Juniperus chinensis* L.	Thailand	T. Trakunyingcharoen	KM006431	KM006462
	CPC22754	*Juniperus chinensis* L.	Thailand	T. Trakunyingcharoen	KM006432	KM006463
Di. olivarum	CBS121887	*Olea europaea* L.	Italy	C. Lazzizera	EU392302	EU392279
	CBS121886	*Olea europaea* L.	Italy	F. Salvatore	EU392301	EU392278
Di. pseudoseriata	CBS124907	*Hexachlamis edulis* O. Berg	Uruguay	C. Perez	EU080922	EU863179
	CBS124906	*Blepharocalyx salicifolius* (Kunth) O.Berg	Uruguay	C. Perez	EU080927	EU863181
Di. quercivora	CBS133852	*Quercus canariensis* Willd.	Tunisia	B.T.Linaldeddu	JX894205	JX894229
	CBS133853	*Quercus canariensis* Willd.	Tunisia	B.T.Linaldeddu	JX894206	JX894230
Di. rosulata	CBS116470	*Prunus africana* (Hook.f.) Kalkman	Ethiopia	A. Gure	EU430265	EU430267
	CBS116472	*Prunus africana* (Hook.f.) Kalkman	Ethiopia	A. Gure	EU430266	EU430268
Di. sapinea	CBS393.84	*Pinus nigra* J.F.Arnold	Netherlands	H.A. van der Aa	DQ458895	DQ458880
	CBS109725	*Pinus patula* Schiede	South Africa	M.J. Wingfield	DQ458896	DQ458881
Di. scrobiculata	CBS109944	*Pinus greggii* Engelm.	Mexico	M.J. Wingfield	DQ458899	DQ458884
	CBS113423	*Pinus greggii* Engelm.	Mexico	M.J. Wingfield	DQ458900	DQ458885
Di. seriata	P5A	*Sorbus intermedia* (Ehrh.) Pers.	Russia	Timur Bulgakov	This work	This work
	P8	*Menispermum canadense* L.	Russia	Timur Bulgakov	This work	This work
	P8ms	*Menispermum canadense* L.	Russia	Timur Bulgakov	This work	This work
	P14A	*Rosa x centifolia* L.	Russia	Timur Bulgakov	This work	This work
	P14B	*Rosa x centifolia* L.	Russia	Timur Bulgakov	This work	This work
	P14-2A	*Rosa x centifolia* L.	Russia	Timur Bulgakov	This work	This work
	P14-2B	*Rosa x centifolia* L.	Russia	Timur Bulgakov	This work	This work
	CBS112555	*Vitis vinífera* L.	Portugal	A.J.L. Phillips	AY259094	AY573220
	CBS119049	*Vitis vinífera* L.	Italy	L. Mugnai	DQ458889	DQ458874
Di. tsugae	CBS418.64	*Tsuga heterophylla* (Raf) Sarg.	British Columbia	A. Funk	DQ458888	DQ458873
Do. americana	CBS128309	*Vitis vinífera* L.	USA	K.Striegler & G.M. Leavitt	HQ288218	HQ288262
	CBS128310	*Vitis vinífera* L.	USA	K.Striegler & G.M. Leavitt	HQ288219	HQ288263

Species	Isolate number	Host	Origin	Collector	Sequences	
					ITS	EF-1α
Do. brevicollis	CMW36463	*Acacia karroo* Hayne	South Africa	F. Jami, M. Gryzenhout	JQ239403	JQ239390
	CMW36464	*Acacia karroo* Hayne	South Africa	F. Jami, M. Gryzenhout	JQ239404	JQ239391
Do. capri-amissi	CBS 121763	*Acacia erioloba* E.Mey.	South Africa	F.J.J. van der Walt	EU101323	EU101368
	CBS121878	*Acacia erioloba* E.Mey	South Africa	F.J.J. van der Walt	EU101324	EU101369
Do. casuarini	CBS120690	*Casuarina* sp.	Australia	M.J. Wingfield	DQ846774	DQ875333
	CBS120688	*Casuarina* sp.	Australia	M.J. Wingfield	DQ846773	DQ875331
Do. dulcispinae	CMW36460	*Acacia karroo* Hayne	South Africa	F. Jami F, M. Gryzenhout	JQ239400	JQ239387
	CMW36462	*Acacia karroo* Hayne	South Africa	F. Jami F, M. Gryzenhout	JQ239402	JQ239389
Do. eriobotryae	BN81	*Eriobotrya japónica* (Thunb.) Lindl.	Spain	E. Gonzalez-Dominguez	KT240287	KT240262
Do. iberica	CAA005	*Pistacia vera* L.	USA	Unknown	EU673312	EU673279
	CBS115041	*Quercus ilex* L.	Spain	A.J.L. Phillips	AY573202	AY573222
Do. iranica	CBS124722	*Olea europaea* L.	Iran	A. Javadi	KC898231	KC898214
Do. longicollis	CMW26165	*Lysiphyllum cunninghamii* (Benth.) de Wit	Australia	T.I. Burgess & M.J. Wingfield	EU144053	EU144068
	CMW26166	*Lysiphyllum cunninghamii* (Benth.) de Wit	Australia	T.I. Burgess & M.J. Wingfield	EU144054	EU144069
Do. moneti	MUCC505	*Acacia rostellifera* Benth.	Australia	K.M. Taylor	EF591920	EF591971
	MUCC506	*Acacia rostellifera* Benth.	Australia	K.M. Taylor	EF591921	EF591972
Do. neclivorem	DAR80992	*Vitis vinífera* L.	Australia	K.M. Taylor	KJ573643	KJ573640
Do. oblonga	CBS121766	*Acacia melífera* (Vahl) Benth	South Africa	F.J.J. van der Walt & R.N. Heath	EU101301	EU101346
	CBS121765	*Acacia melífera* (Vahl) Benth	South Africa	F.J.J. van der Walt & R.N. Heath	KF766163	EU101345
Do. omnivora	P9A	*Juglans regia* L.	Russia	Timur Bulgakov	This work	This work
	P9B	*Juglans regia* L.	Russia	Timur Bulgakov	This work	This work
	P9C	*Juglans regia* L.	Russia	Timur Bulgakov	This work	This work
	P9D	*Juglans regia* L.	Russia	Timur Bulgakov	This work	This work
	P15-2A ms	*Cotoneaster melanocarpus* G. Lodd.	Russia	Timur Bulgakov	This work	This work
	P15-2B ms	*Cotoneaster melanocarpus* G. Lodd.	Russia	Timur Bulgakov	This work	This work
	P28-2	*Acer negundo* L.	Russia	Timur Bulgakov	This work	This work
	P28-2ms	*Acer negundo* L.	Russia	Timur Bulgakov	This work	This work

Species	Isolate number	Host	Origin	Collector	Sequences	
					ITS	EF-1α
	P39 ms	*Fraxinus pennsylvanica* Marshall	Russia	Timur Bulgakov	This work	This work
	P39-2A	*Fraxinus pennsylvanica* Marshall	Russia	Timur Bulgakov	This work	This work
	P39-2B	*Fraxinus pennsylvanica* Marshall	Russia	Timur Bulgakov	This work	This work
	P39-2C	*Fraxinus pennsylvanica* Marshall	Russia	Timur Bulgakov	This work	This work
	P67-1	*Cornus sanguinea* L.	Russia	Timur Bulgakov	This work	This work
	P67-2	*Cornus sanguinea* L.	Russia	Timur Bulgakov	This work	This work
	P78	*Sorbus aucuparia* L.	Russia	Timur Bulgakov	This work	This work
	P94	*Parthenocissus quinquefolia* (L.) Planch.	Russia	Timur Bulgakov	This work	This work
	CBS188.87	*Juglans regia* L.	France	Meylan	EU673316	EU673283
	CBS242.51	*Unknown*	Italy	R. Ciferri	EU673317	EU673284
Do. parva	CBS124720	*Corylus avellana* L.	Iran	J. Abdollahzadeh/A. Javadi	KC898234	KC898217
	CBS124721	*Corylus avellana* L.	Iran	J. Abdollahzadeh/A. Javadi	KC898235	KC898218
Do. pretoriensis	CMW36480	*Acacia karroo* Hayne	South Africa	Jami, Gryzenh.	JQ239405	JQ239392
	CMW36481	*Acacia karroo* Hayne	South Africa	Jami, Gryzenh.	JQ239406	JQ239393
Do. prunicola	CBS124723	*Prunus dulcis* Mill.	Portugal	E. Diogo	EU673313	EU673280
Do. rhamni	P83	*Rhamnus cathartica* L.	Russia	Timur Bulgakov	This work	This work
Do. santali	MUCC509	*Santalum acuminatum* DC.	Australia	T.I. Burgess	EF591924	EF591975
	MUCC508	*Santalum acuminatum* DC.	Australia	T.I. Burgess	EF591923	EF591974
Do. sarmentorum	P1-2B	*Crataegus crus-gallii* L.	Russia	Timur Bulgakov	This work	This work
	P1-2C	*Crataegus crus-gallii* L.	Russia	Timur Bulgakov	This work	This work
	P1-2D	*Crataegus crus-gallii* L.	Russia	Timur Bulgakov	This work	This work
	P2-2A	*Sorbus aucuparia* L.	Russia	Timur Bulgakov	This work	This work
	P3-2A	*Euomymus europaea* L.	Russia	Timur Bulgakov	This work	This work
	P3-2B	*Euomymus europaea* L.	Russia	Timur Bulgakov	This work	This work
	P4-2A	*Prunus armeniaca* L.	Russia	Timur Bulgakov	This work	This work
	P4-2B	*Prunus armeniaca* L.	Russia	Timur Bulgakov	This work	This work
	P6A	*Cornus sanguinea* L.	Russia	Timur Bulgakov	This work	This work

Species	Isolate number	Host	Origin	Collector	Sequences	
					ITS	EF-1α
	P6B	*Cornus sanguínea* L.	Russia	Timur Bulgakov	This work	This work
	P7A	*Tamarix ramosissima* Ledeb.	Russia	Timur Bulgakov	This work	This work
	P7B	*Tamarix ramosíssima* Ledeb.	Russia	Timur Bulgakov	This work	This work
	P8A	*Menispermum canadense* L.	Russia	Timur Bulgakov	This work	This work
	P8B	*Menispermum canadense* L.	Russia	Timur Bulgakov	This work	This work
	P10-2A	*Juglans regia* L.	Russia	Timur Bulgakov	This work	This work
	P10-2B	*Juglans regia* L.	Russia	Timur Bulgakov	This work	This work
	P11-2A	*Cornus sanguinea* L.	Russia	Timur Bulgakov	This work	This work
	P11-2B	*Cornus sanguínea* L.	Russia	Timur Bulgakov	This work	This work
	P12A	*Cornus sanguínea* L.	Russia	Timur Bulgakov	This work	This work
	P13B	*Syringa vulgaris* L.	Russia	Timur Bulgakov	This work	This work
	P18-2	*Celastrus orbiculatus* Thunb.	Russia	Timur Bulgakov	This work	This work
	P19A	*Salix babylonica* L.	Russia	Timur Bulgakov	This work	This work
	P19B	*Salix babylonica* L.	Russia	Timur Bulgakov	This work	This work
	P34A	*Parthenocissus quinquefolia* (L.) Planch	Russia	Timur Bulgakov	This work	This work
	P34B	*Parthenocissus quinquefólia* (L.) Planch	Russia	Timur Bulgakov	This work	This work
	P37 ms	*Acer negundo* L.	Russia	Timur Bulgakov	This work	This work
	P37-2A	*Acer negundo* L.	Russia	Timur Bulgakov	This work	This work
	P37-2B	*Acer negundo* L.	Russia	Timur Bulgakov	This work	This work
	P37-2C	*Acer negundo* L.	Russia	Timur Bulgakov	This work	This work
	P40A	*Styphnolobium japonicum* (L.) Schott	Russia	Timur Bulgakov	This work	This work
	P40B	*Styphnolobium japonicum* (L.) Schott	Russia	Timur Bulgakov	This work	This work
	P46A	*Lycium barbarum* L.	Russia	Timur Bulgakov	This work	This work
	P46B	*Lycium barbarum* L.	Russia	Timur Bulgakov	This work	This work
	P50A	*Cornus sanguinea* L.	Russia	Timur Bulgakov	This work	This work
	P50B	*Cornus sanguinea* L.	Russia	Timur Bulgakov	This work	This work
	P64A	*Fraxinus pennsylvanica* Marshall	Russia	Timur Bulgakov	This work	This work

Species	Isolate number	Host	Origin	Collector	Sequences	
					ITS	EF-1α
	P64B	*Fraxinus pennsylvanica* Marshall	Russia	Timur Bulgakov	This work	This work
	P65A	*Acer tataricum* L.	Russia	Timur Bulgakov	This work	This work
	P65B	*Acer tataricum* L.	Russia	Timur Bulgakov	This work	This work
	P69A	*Morus alba* L.	Russia	Timur Bulgakov	This work	This work
	P69B	*Morus alba* L.	Russia	Timur Bulgakov	This work	This work
	P76-1	*Prunus fruticose* Pall.	Russia	Timur Bulgakov	This work	This work
	P76-2	*Prunus fruticose* Pall.	Russia	Timur Bulgakov	This work	This work
	P79-1	*Cotoneaster* sp.	Russia	Timur Bulgakov	This work	This work
	P79-2	*Cotoneaster* sp.	Russia	Timur Bulgakov	This work	This work
	P81	*Fraxinus pennsylvanica* L.	Russia	Timur Bulgakov	This work	This work
	P85	*Pyrus communis* L.	Russia	Timur Bulgakov	This work	This work
	P87	*Acer tataricum* L.	Russia	Timur Bulgakov	This work	This work
	P92-1	*Cornus sanguinea* L.	Russia	Timur Bulgakov	This work	This work
	IMI63581b	*Ulmus* sp.	England	E. A. Ellis	AY573212	AY573235
	CBS115038	*Malus pumila* Miller	Netherlands	A.J.L. Phillips	AY573206	AY573223
Do. sempervirentis	CBS124718	*Cupressus sempervirens* L.	Iran	M.A. Aghajani	KC898236	KC898219
	CBS124719	*Cupressus sempervirens* L.	Iran	M.A. Aghajani	KC898237	KC898220
Do. striata	CBS124730	*Citrus sinensis* (L.) Osbeck	New Zealand	S.R. Pennycook/P.R. Johnston	EU673320	EU673287
	CBS124731	*Citrus sinensis* (L.) Osbeck	New Zealand	S.R. Pennycook/P.R. Johnston	EU673321	EU673288
Do. symphoricarposicola	MFLUCC13-0497	*Symphoricarpos* sp.	Italy	Erio Camporesi	KJ742378	KJ742381
	MFLUCC13-0498	*Symphoricarpos* sp.	Italy	Erio Camporesi	KJ742379	KJ742382
Do. thailandica	CBS133991	*Bambusa* sp.	Thailand	Dongqin Dai	JX646796	JX646861
Do. thripsita	BRIP51876	*Acacia harpophylla* F.Muell.	Australia	D.J. Tree & C.E.C. Tree	KJ573642	KJ573639
Do. uruguayensis	UY672	*Hexachlamis edulis* O. Berg	Uruguay	C. Perez	EU080923	EU863180
Do. vidmadera	DAR78992	*Vitis vinífera* L.	Australia	Pitt & Loschiavo	EU768874	EU768881
	DAR78994	*Vitis vinífera* L.	Australia	Pitt & Loschiavo	EU768877	EU768883
Do. vinea	DAR81012	*Vitis vinífera* L.	Australia	N. Wunderlich	KJ573644	KJ573641

Species	Isolate number	Host	Origin	Collector	Sequences	
					ITS	EF-1α
P. cupressi	IRAN1455C	*Cupressus semipervirens* L.	Iran	J. Abdollahzadeh	FJ919672	FJ919661
	IRAN1458C	*Cupressus semipervirens* L.	Iran	J. Abdollahzadeh	FJ919671	FJ919660
P. mamane	CPC 12440	*Sophora chrysophylla* (Salisb.) Seem	USA	A.J.L. Phillips	EU673332	EU673297
	CPC 12442	*Sophora chrysophylla* (Salisb.) Seem	USA	A.J.L. Phillips	EU673333	EU673298
P. negundinis	P17-2 A	*Ligustrum vulgare* L.	Russia	Timur Bulgakov	This work	This work
	P25B	*Acer negundo* L.	Russia	Timur Bulgakov	This work	This work
	P35-2ms	*Acer negundo* L.	Russia	Timur Bulgakov	This work	This work
	P75-1	*Forsythia* × *intermedia* Zabel	Russia	Timur Bulgakov	This work	This work
	P75-2	*Forsythia* × *intermedia* Zabel	Russia	Timur Bulgakov	This work	This work
P. rhois	CFCC 89663	*Rhus typhina* L.	China	Hong Fan	KM030585	KM030599
	CFCC 89662	*Rhus typhina* L.	China	Hong Fan	KM030584	KM030598
S. viticola	CBS117009	*Vitis vinífera* L.	Spain	J. Luque & S. Martos	AY905554	AY905559
	CBS302.75	*Poniciana gilliesii* Wallich	France	M. Morelet	EU673319	EU673286

Abbreviations: CAA: Collection of Artur Alves housed at Department of Biology, University of Aveiro, Portugal; CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; CMW: FABI, University of Pretoria, South Africa; CPC Collection of Pedro Crous housed at CBS; MFLUCC: Mae Fah Luang University Culture Collection, Chiang Rai, Thailand.

Table 4 – Hosts from which the isolates were obtained as well as first registered occurrence for the species and location.

Species	Host(s)	Location
D. intermedia	*Sorbus aucuparia* L.*	Rostov-on-Don city
	Sorbus intermedia (Ehrh.) Pers.*	Rostov-on-Don city
D. seriata	*Menispermum canadense* L.*	Rostov-on-Don city
	Rosa × *centifolia* L.	Rostov-on-Don city
Do. omnivora	*Acer negundo* L.*	Rostov-on-Don city
	Cornus sanguinea L.*	Krasnosulinsky district
	Cotoneaster laxiflorus J. Jacq. ex Lindl.*	Rostov-on-Don city
	Fraxinus pennsylvanica Marshall*	Rostov-on-Don city
	Juglans regia L.	Rostov-on-Don city
	Sorbus aucuparia L.*	Krasnosulinsky district
Do. rhamni	*Rhamnus cathartica* L.	Oktyabrsky district
Do. sarmentorum	*Acer negundo* L.*	Shakhty city
	Acer tataricum L.*	Oktyabrsky district
	Celastrus orbiculatus Thunb.*	Rostov-on-Don city
	Cotoneaster sp.*	Rostov-on-Don city
	Cornus sanguinea L.*	Shakhty city
	Cornus sanguinea L.	Krasnosulinsky district
	Crataegus crus-galli L.	Rostov-on-Don city
	Euonymus europaeus L.	Rostov-on-Don city
	Fraxinus pennsylvanica Marshall*	Oktyabrsky district
	Fraxinus pennsylvanica Marshall	Shakhty city
	Juglans regia L.*	Shakhty city
	Lycium barbarum L.*	Shakhty city
	Menispermum canadense L.	Rostov-on-Don city
	Morus alba L.	Shakhty city
	Parthenocissus quinquefolia (L.) Planch.*	Shakhty city
	Prunus armeniaca L.	Shakhty city
	Prunus fruticosa Pall.*	Shakhty city
	Pyrus communis L.	Shakhty city
	Tamarix ramosissima Ledeb.*	Shakhty city
	Salix babylonica L.*	Shakhty city
	Sorbus aucuparia L.*	Rostov-on-Don city
	Styphnolobium japonicum (L.) Schott*	Rostov-on-Don city
	Syringa vulgaris L.*	Shakhty city

* First occurrence in this species host.

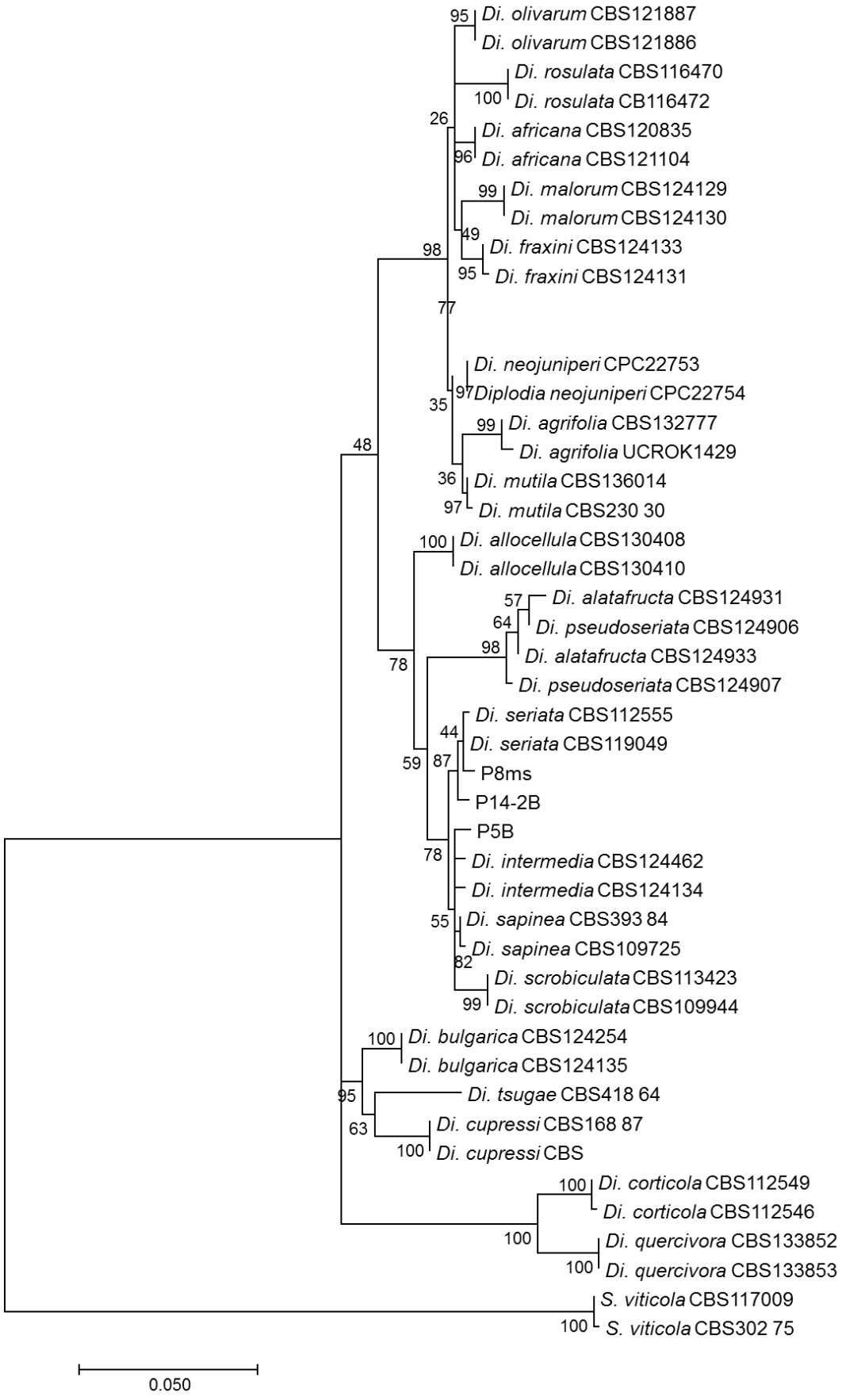
95 Di. olivarum CBS121887
Di. olivarum CBS121886
Di. rosulata CBS116470
100 Di. rosulata CB116472
26
Di. africana CBS120835
96 Di. africana CBS121104
99 Di. malorum CBS124129
Di. malorum CBS124130
49
98 Di. fraxini CBS124133
95 Di. fraxini CBS124131
77
Di. neojuniperi CPC22753
97 Diplodia neojuniperi CPC22754
35
99 Di. agrifolia CBS132777
48 Di. agrifolia UCROK1429
36 Di. mutila CBS136014
97 Di. mutila CBS230 30
100 Di. allocellula CBS130408
Di. allocellula CBS130410
57 Di. alatafructa CBS124931
64 Di. pseudoseriata CBS124906
98 Di. alatafructa CBS124933
78 Di. pseudoseriata CBS124907
Di. seriata CBS112555
44 Di. seriata CBS119049
59 87 P8ms
P14-2B
P5B
78 Di. intermedia CBS124462
Di. intermedia CBS124134
55 Di. sapinea CBS393 84
Di. sapinea CBS109725
82 Di. scrobiculata CBS113423
99 Di. scrobiculata CBS109944
100 Di. bulgarica CBS124254
Di. bulgarica CBS124135
95 Di. tsugae CBS418 64
63 Di. cupressi CBS168 87
100 Di. cupressi CBS
100 Di. corticola CBS112549
Di. corticola CBS112546
100 Di. quercivora CBS133852
100 Di. quercivora CBS133853
S. viticola CBS117009
100 S. viticola CBS302 75
0.050

Figure 2 – ITS-TEF ML tree for *Diplodia* genus - The evolutionary history was inferred by using the Maximum Likelihood method based on the General Time Reversible model (Rodríguez et al., 1990). The tree with the highest log likelihood (-2929.4775) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (6 categories (+*G*, parameter = 0.3002)). The rate variation model allowed for some sites to be evolutionarily invariable ([+*I*], 38.2831% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 46 nucleotide sequences. There were a total of 862 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016).

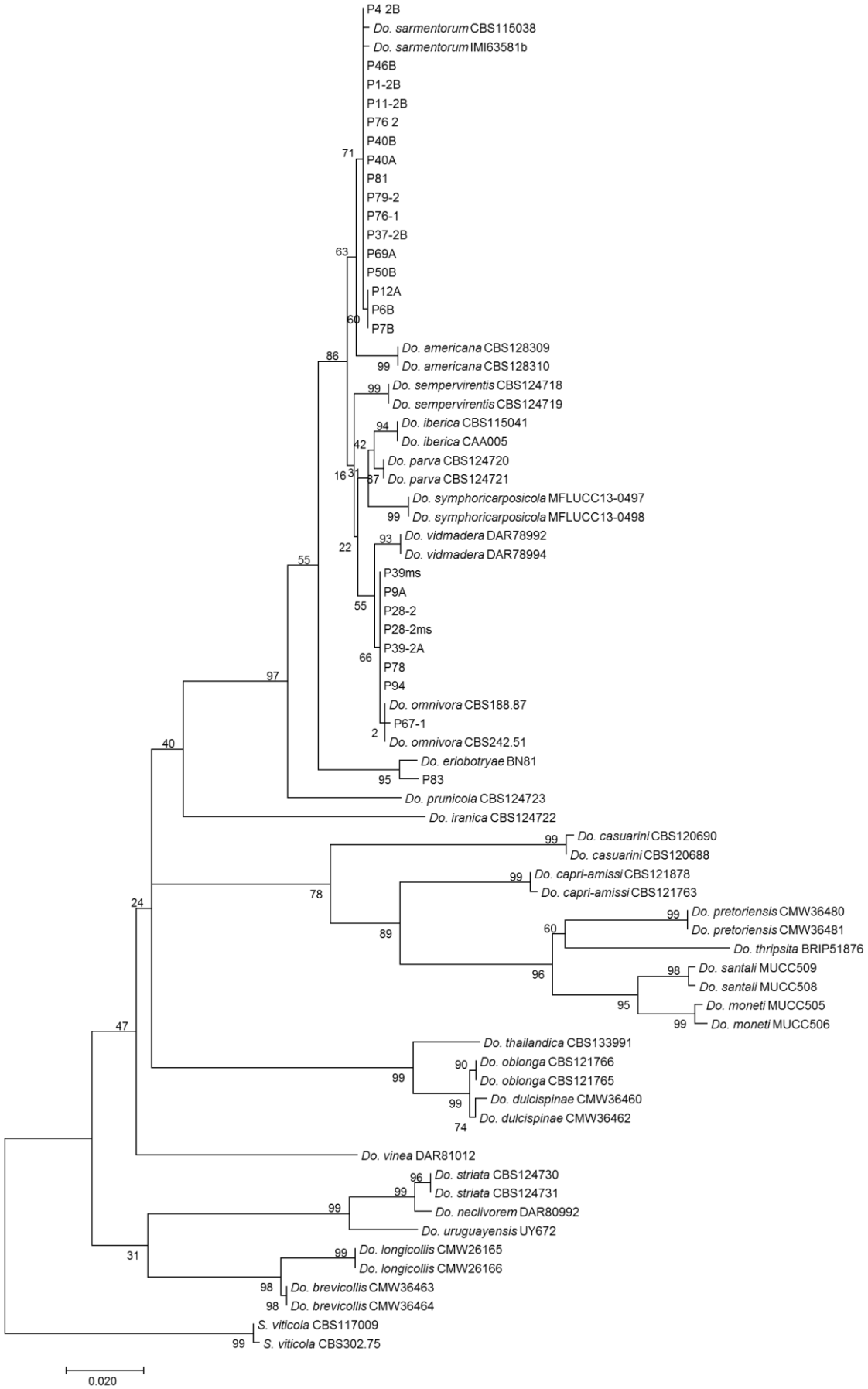
P4 2B
Do. sarmentorum CBS115038
Do. sarmentorum IMI63581b
P46B
P1-2B
P11-2B
P76 2
P40B
P40A
P81
P79-2
P76-1
P37-2B
P69A
P50B
P12A
P6B
P7B
Do. americana CBS128309
Do. americana CBS128310
Do. sempervirentis CBS124718
Do. sempervirentis CBS124719
Do. iberica CBS115041
Do. iberica CAA005
Do. parva CBS124720
Do. parva CBS124721
Do. symphoricarposicola MFLUCC13-0497
Do. symphoricarposicola MFLUCC13-0498
Do. vidmadera DAR78992
Do. vidmadera DAR78994
P39ms
P9A
P28-2
P28-2ms
P39-2A
P78
P94
Do. omnivora CBS188.87
P67-1
Do. omnivora CBS242.51
Do. eriobotryae BN81
P83
Do. prunicola CBS124723
Do. iranica CBS124722
Do. casuarini CBS120690
Do. casuarini CBS120688
Do. capri-amissi CBS121878
Do. capri-amissi CBS121763
Do. pretoriensis CMW36480
Do. pretoriensis CMW36481
Do. thripsita BRIP51876
Do. santali MUCC509
Do. santali MUCC508
Do. moneti MUCC505
Do. moneti MUCC506
Do. thailandica CBS133991
Do. oblonga CBS121766
Do. oblonga CBS121765
Do. dulcispinae CMW36460
Do. dulcispinae CMW36462
Do. vinea DAR81012
Do. striata CBS124730
Do. striata CBS124731
Do. neclivorem DAR80992
Do. uruguayensis UY672
Do. longicollis CMW26165
Do. longicollis CMW26166
Do. brevicollis CMW36463
Do. brevicollis CMW36464
S. viticola CBS117009
S. viticola CBS302.75
0.020

Figure 3 - ITS-TEF ML tree for *Dothiorella* genus - The evolutionary history was inferred by using the Maximum Likelihood method based on the General Time Reversible model (Rodríguez et al., 1990). The tree with the highest log likelihood (-4228.5537) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (6 categories (+G, parameter = 0.2553)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 39.6847% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 72 nucleotide sequences. There were a total of 1110 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016).

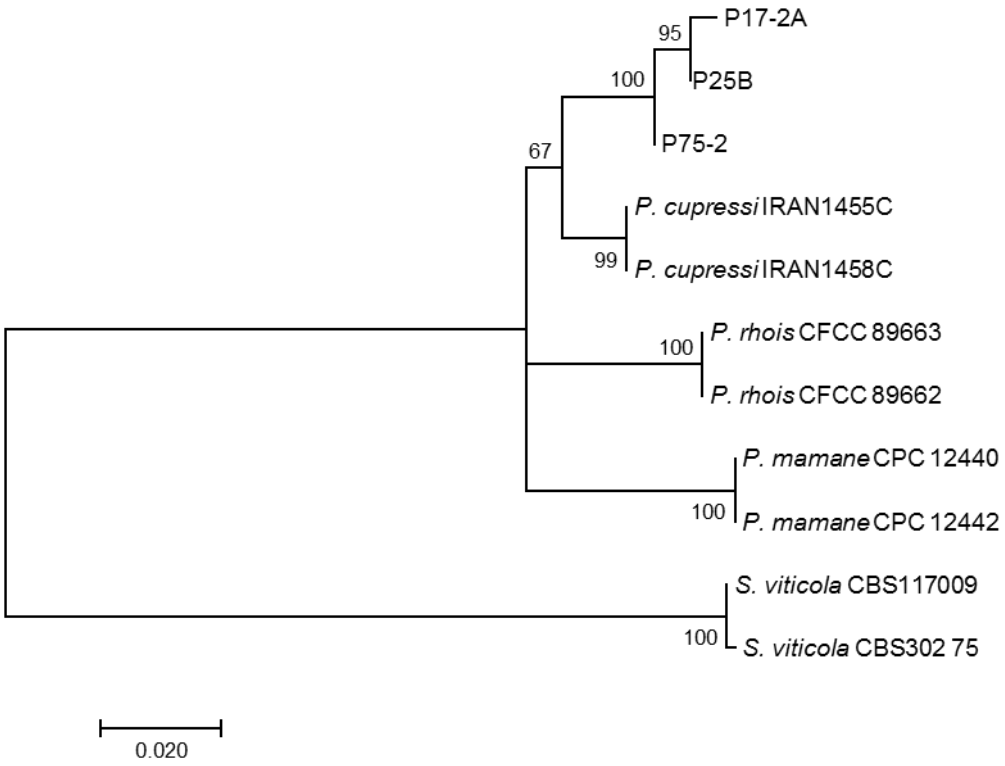

Figure 4 – ITS-TEF ML tree for *Phaeobotryon* genus - The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). The tree with the highest log likelihood (-1901.3344) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (6 categories (+G, parameter = 0.2378)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 41.7582% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 11 nucleotide sequences. There were a total of 819 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016).

4.4 - Morphological characterisation

4.4.1 - P 83 –*Dothiorella rhamni* Wanasinghe, Bulgakov, E.B.G. Jones & K.D. Hyde, sp. nov. (Figure 5).

Index Fungorum number: IF 551784

Etymology: Name comes from the host genus name *Rhamnus*, from which it was isolated for the first time.

Conidiomata pycnidial, produced on pine needles on ¼ strength PDA, within 3 to 4 weeks, normally individual, superficial and/or semi-immersed, covered with hyphal hairs uniloculate and thick walled. The walls are composed of *textura angularis* with a few layers starting with a brown colour going degrade until it becomes hyaline when it reaches the conidiogenous tissue. Conidiogenous cells (A,B) were cylindrical to ovoid shape, discrete or integrated holoblastic indeterminate proliferating at the same level originating periclinal thickenings, hyaline, thin walled and smooth. Conidia (C,D) ellipsoid, becoming light brown and 1-septate after being detached from conidiogenous tissue, sometimes have a slight constriction around septum, thick walls and smooth surface, ends are normally rounded.

Conidial dimensions: (19.88-) 23.24 - 23.81 (-27.16) x (7.86-) 9.10 - 9.32 (-10.74) µm. Average L/W = 2.56 ± 0.2.

Culture characteristics – cottony culture like with whitish to pearl colour, becoming completely dark olivaceous green over time with a lot of aerial mycelium and just the same aspect on reverse of the plate. Culture reached 42 ± 6.68 mm after 5 days in the dark at 25 ºC which was the temperature that registered the biggest growth rate.

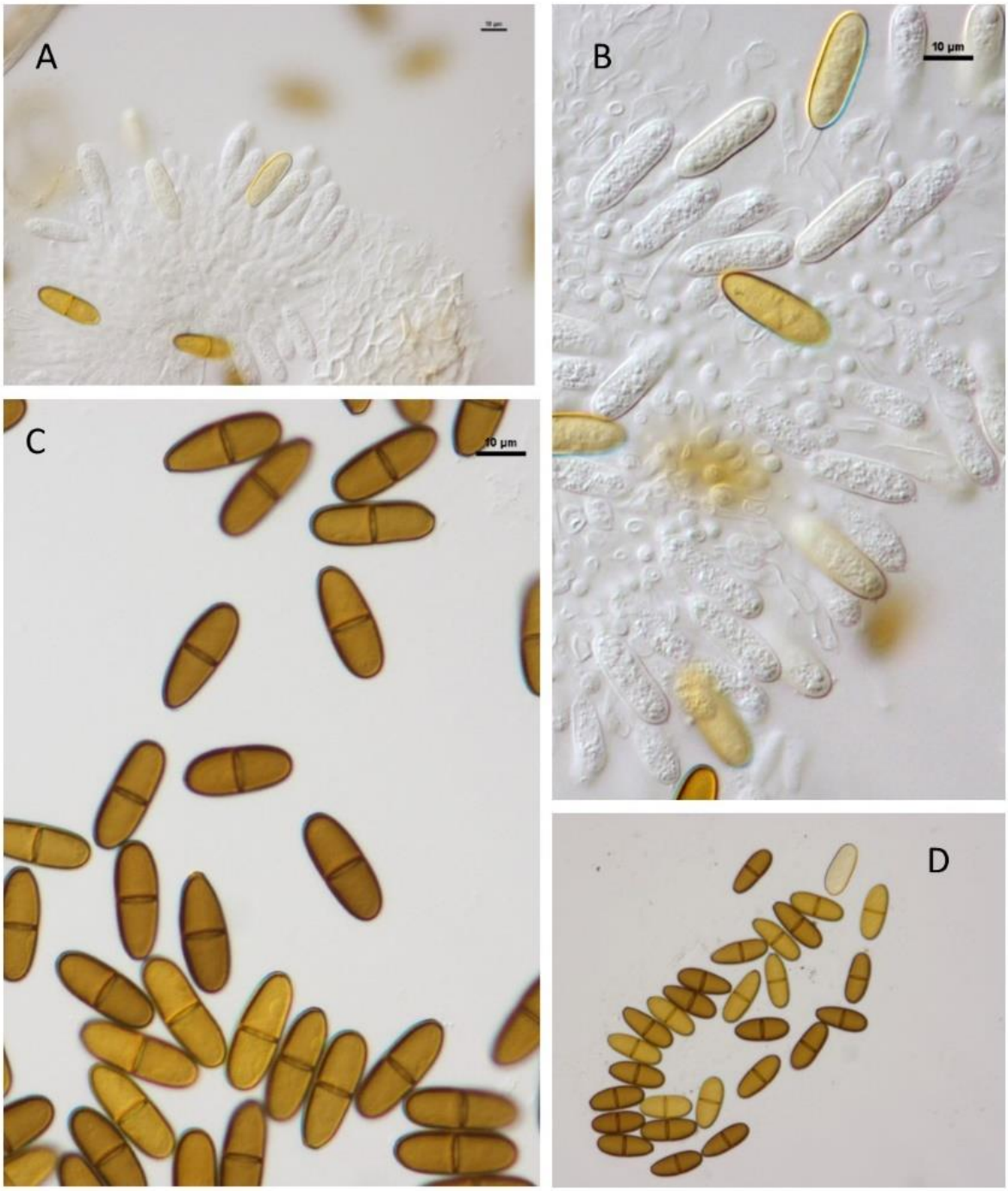

Figure 5 – P 83 isolate *Dothiorella rhamni*. A-B. Young conidiogenous cells with developing conidia C-D. Matured 1-peptate conidia. Scale bars: only apply to B and C.

4.4.2 - P 25B/ P 75-2/ P 17-2A - *Phaeobotryon negundinis* Daranagama, Bulgakov and K.D. Hyde, sp. nov. (Figure 6)

Index Fungorum number: IF551954

Etymology: first isolated from *Acer negundo*,"*negundinis*" refers to host.

Conidiomata pycnidial (A,B), produced on pine needles on ¼ strength PDA within 3 to 4 weeks, normaly individual, superficial and semi-immersed, covered with hyphal hairs uniloculate and thick walled. The walls are composed of *textura angularis* with a few layers starting with a brown colour going degrade until it becomes hyaline when it reaches the conidiogenous tissue. Conidiogenous cells (C,D) are cylindrical to ovoid shape, discrete or integrated holoblastic indeterminate proliferating at the same level originating periclinal thickenings, hyaline, thin walled and smooth. Conidia (E-J) subcylindrical to ellipsoid, brown without septum, thick walled and smooth surface, ends normally rounded but often with a tapered base.

Conidia dimensions: (13.72-) 21.57 - 22.35 (-27.04) x (6.83-) 7.71 - 7.89 (-9.16) µm.

Average L/W = 2.82 ± 0.27.

Culture characteristics – cottony culture like with whitish to pearl colour, becoming completely dark olivaceous green becoming almost black over time with a lot of aerial mycelium the reverse of the plate had the same aspect. Culture reached 47 ± 9.2 mm after 5 days in the dark at 25 ºC which was the temperature that registered the biggest growth rate.

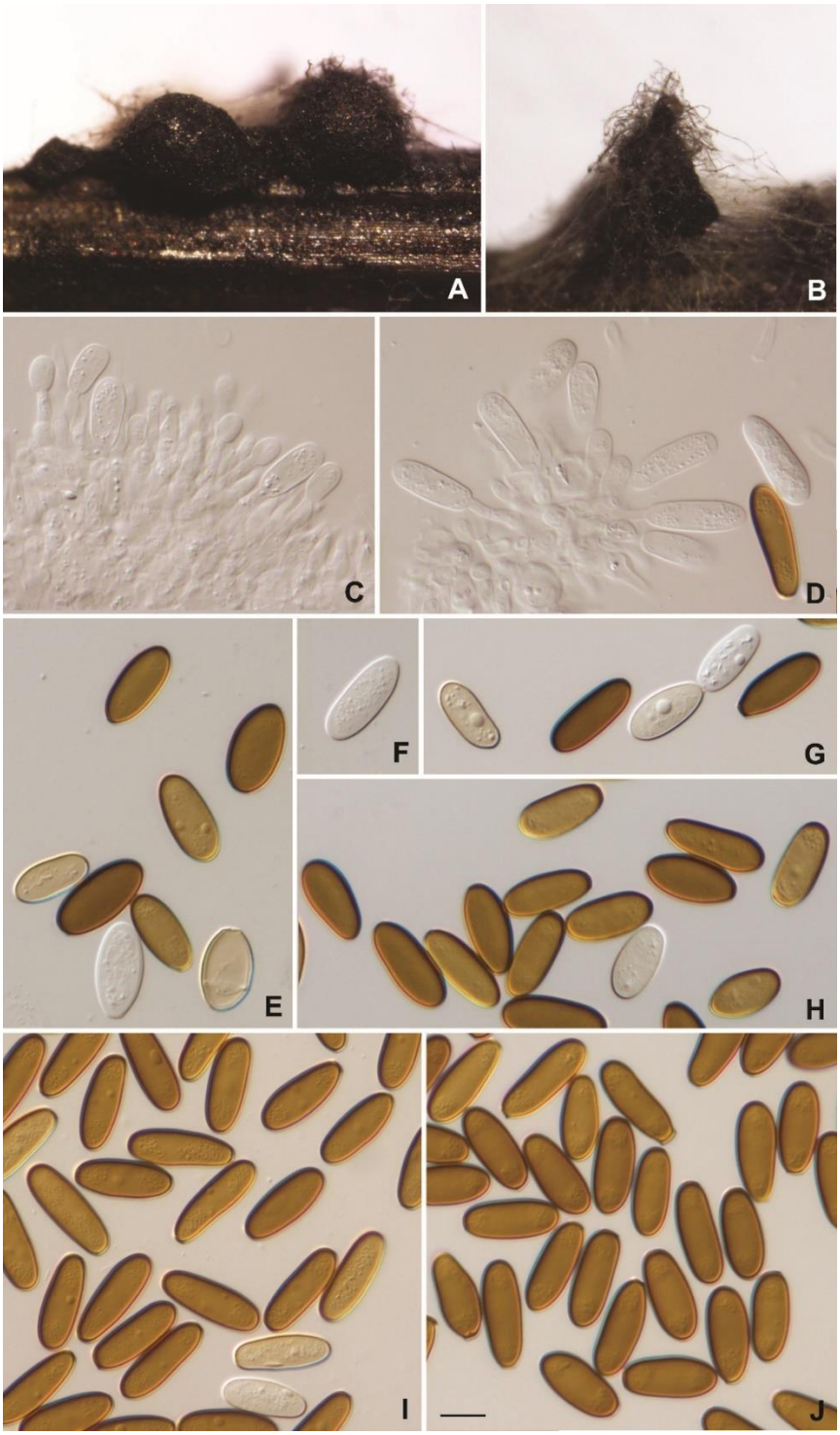

Figure 6 - P25B isolate *Phaeobotryon negundinis* (new species) – A, C. Conidiomata formed on pine needles in culture. C, D. Conidia on conidiogenous cells. F. Hyaline immature conidia. E, G, H. Mature and hyaline aseptate conidia. Scale bar: J = 10 µm applies for C-J.

5. - Discussion

For this study 25 different plant hosts species from Rostov, Russia, were sampled to assess the diversity of *Botryosphaeriaceae* fungi. After the isolation and isolate cultures were established the first approach was based on BOX-PCR fingerprinting, which rapidly and efficiently analysed all isolates, dividing them in 3 major clusters with low similarity corresponding to different genera, with the most frequent being *Dothiorella* with 83% (n=67), followed by *Diplodia* with 11% (n=9) and *Phaeobotryon* with 6% (n=5) .

The genus *Dothiorella* has been reported worldwide, consult SMML Fungus-Host Distribution Database (Farr and Rossman, 2016). *Dothiorella* species were isolated from several woody hosts, both gymnosperms and angiosperms, and they are usually saprophytes or weak pathogens. The majority of the isolates belonged to the species *Dothiorella sarmentorum* 61% (n=49*)*, being one of the most dispersed species with reports from all continents and a very wide range of hosts such as *Malus pumila*, *Menispersum canadense*, *Prunus armeniaca*, *Prunus dulcis*, *Pyrus communis*, *Ulmus* sp. *Vitis vinifera* and many others (Phillips et al., 2013).

Dothiorella omnivora was the second most prevalent and abundant with 20% (n=16), and isolated from several different hosts. This species was recently described from hazelnut and several other hosts; the name given is originated in the wide range of hosts used by it (Linaldeddu et al., 2016). *Dothiorella* are weak pathogens and for the time being remains to be established the exact role in cankers on hazelnut and as an endophyte (Linaldeddu et al., 2016).

The species *Do. sarmentorum* and *Do. omnivora* are reported here for the first time from Russia and regarding *Do. sarmentorum* this is the first report on 15 different host species (Table 3), *Dothiorella sarmentorum* has an amazingly wide host range. *Do. omnivora* is reported for the first time in *Acer negundo* L. *Cornus sanguinea* L. *Cotoneaster laxiflorus* J. Jacq. ex Lindl. *Fraxinus pennsylvanica* Marshall and *Sorbus aucuparia* L..

Dothiorella rhamni, a new species of *Dothiorella*, represented 1% (n=1) of total isolates a description is given in this work but was first described in another one (Li et al., 2016). The given name comes from the host genus name, from which it was isolated, *Rhamnus cathartica* L. (Li et al., 2016).

Species of *Diplodia* are widely known, worldwide for being pathogens, saprophytes and endophytic from both gymnosperms and angiosperms. Two serious pathogens from this genus are *Diplodia sapinea* and *Diplodia seriata*, both are known to cause crown wilt, dieback, shoot blight, frog-eye leaf spot, black rot, and cankers (Crous et al., 2006; Phillips et al., 2012; Slippers and Wingfield, 2007).

Two species of *Diplodia* were identified, *Diplodia seriata* corresponding to 9% (n=7) of isolates and *Diplodia intermedia* corresponding to 3% (n=2) of total isolates. *Diplodia seriata* is a species whose host range includes a wide diversity of angiosperms and gymnosperms, and have been reported from more than 30 different hosts, some of those are, *Cedrus deodara*, *Citrus limon*, *Picea glauca*, *Protea longifolia*, *Prunus domestica*, *Pyrus communis*, *Vitis vinifera* and many more. The second species identified was *Diplodia intermedia, which* has been reported from Portugal in three different hosts, *Cydonia* sp.*, Malus domestica* and *Malus sylvestris,* in France on *Vitis vinifera* and on *Malus* sp*.* in Uruguay. (Comont et al., 2016; Delgado-Cerrone et al., 2016; Phillips et al., 2012).

The species *Di. seriata* and *Di. intermedia* are reported here for the first time from Russia and regarding *Di. seriata* this is the first report of the species on *Menispermum canadense* L. and for *Di. intermedia* is the first report from *Sorbus aucuparia* L. and *Sorbus intermedia* (Ehrh.) Pers.

The genus *Phaeobotryon* comprises at the moment three species known form culture, *P. cupressi*, *P. mamane* and *P. rhois* (Abdollahzadeh et al., 2009; Fan et al., 2015; Phillips et al., 2013). All these species were collected from diseased plant tissue and is obviously a growing genus in terms of diversity. So far these species have been recorded from *Cupressus sempervirens* L., *Juniperus scopulorum* Sargent, *Quercus* sp. and *Sophora chrysophylla* (Salisb.) *Rhus typhina* L. It was only found one species of this genus, a novel species, since it did

not group with any other. *Phaebotryon negundinis* comprised 6% (n=5) of total isolates. This species can be distinguished phylogenetically from all other species due to its smaller conidia and no sexual morph was found until now. This work also reports for the first time a *Phaebotryon* species on *Acer negundo*, *Forsythia* x *intermedia* and also *Ligustrum vulgare*. (Daranagama et al. 2016, this work). Remain to be studied the true role of *Phaeobotryon* species as endophytic, since all isolation were made from dead plant tissue or from symptomatic hosts, isolating these species from healthy hosts could help to unveil the role of this genus as possible opportunistic pathogens.

6. – Conclusions

In summary the objectives established were met, in terms of diversity three genera in the family of *Botryosphaeriaceae* were found with two of the total 6 species being new to science until this year and also described in this work, many new host affiliations were established for many species as well as the new geographical range.

This work could advance to a second phase to study the pathogenicity of the isolates, adding valuable information about the potential of these species to become serious pathogens. Pathogenicity assays performed on *Eucalyptus globulus* using isolates from *Diplodia* and *Neofusicoccum* genus (Barradas et al., 2016) would fit as a methodology to follow on a later work.

7. - References

Abdollahzadeh, J., Goltapeh, E.M., Javadi, A., Shams-bakhsh, M., Zare, R., Phillips, A.J.L., 2009. *Barriopsis iraniana* and *Phaeobotryon cupressi*: two new species of the *Botryosphaeriaceae* from trees in Iran. Persoonia - Molecular Phylogeny and Evolution of Fungi 23, 1–8.

Ahumada, R., 2003. Pathogens in commercial *Eucalyptus* plantations in Chile, with special reference to *Mycosphaerella* and *Botryosphaeria* species. Faculty of Natural and Agricultural Science of University of Pretoria.

Alves, A, Crous, P.W., Correia, A., Phillips, A J.L., 2008. Morphological and molecular data reveal cryptic speciation in *Lasiodiplodia theobromae*. Fungal Diversity 28, 1–13.

Alves, A., Correia, A., Luque, J., Phillips, A., 2004. *Botryosphaeria corticola*, sp. nov. on Quercus species, with notes and description of *Botryosphaeria stevensii* and its anamorph, *Diplodia mutila*. Mycologia 96, 598–613.

Alves, A., Phillips, A.J.L., Henriques, I., Correia, A., 2007. Rapid differentiation of species of *Botryosphaeriaceae* by PCR fingerprinting. Research in Microbiology 158, 112–121.

Arx, J., Müller, E., 1954. Die amerosporen Gattungen der Pyrenomyceten. Beitrage zur Kryptogamenflora der Schweiz 11, 1–434.

Barradas, C., Phillips, A.J.L., Correia, A., Diogo, E., Bragança, H., Alves, A., 2016. Diversity and potential impact of *Botryosphaeriaceae* species associated with *Eucalyptus globulus* plantations in Portugal. European Journal of Plant Pathology 1–13.

Bega, R. V., Smith, R.S.J., Martinez, A.P., Davis, C.J., 1978. Severe damage to *Pinus radiata* and *P. pinaster* by *Diplodia pinea* and *Lophodermium* spp. on Molokai and Lanai in Hawaii. Plant Disease 62, 311–329.

Blodgett, J., Kruger, E., Stanosz, G., 1997. *Sphaeropsis sapinea* and water stress in a red pine plantation in central Wisconsin. Phytopathology 87, 429–434.

Brown, II, E.A., 1981. Pathogenicity and Histopathology of *Botryosphaeria dothidea* on Apple Stems. Phytopathology 71, 375–379.

Brown, E., Britton, K., 1986. *Botryosphaeria* diseases of apple and peach in the southeastern United States. Plant Disease 70, 480–484.

Carroll, G., 1988. Fungal endophytes in stems and leaves: from latent pathogen to mutualistic symbiont. Ecology 69, 2–9.

Carroll, G.C., 1990. Fungal endophytes in vascular plants: mycological research opportunities in Japan. Transactions of the Mycological Society of Japan 31, 103–116.

Cilliers, A., Swart, W., Wingfield, M., 1995. The occurrence of *Lasiodiplodia theobromae* on *Pinus elliottii* seeds in South Africa. Seed science and technology 23, 851–860.

Comont, G., Mayet, V., Corio-Costet, M., 2016. First Report of *Lasiodiplodia viticola*, *Spencermartinsia viticola* and *Diplodia intermedia* Associated with *Vitis vinifera* Grapevine Decline in French Vineyards. Plant Disease.

Crous, P.W., Müller, M.M., Sánchez, R.M., Giordano, L., Bianchinotti, M.V., Anderson, F.E., Groenewald, J.Z., 2015. Resolving *Tiarosporella spp.* allied to Botryosphaeriaceae and Phacidiaceae. Phytotaxa 202, 73.

Crous, P.W., Slippers, B., Wingfield, M.J., Rheeder, J., Marasas, W.F.O., Philips, A.J.L., Alves, A., Burgess, T., Barber, P., Groenewald, J.Z., 2006. Phylogenetic lineages in the *Botryosphaeriaceae*. Studies in Mycology 55, 235–253.

Daranagama, D., Thambugala, K., Campino, B., Alves, A., Bulgakov, T., Phillips, A., Liu, X., Hyde, K., 2016. *Phaeobotryon negundinis* sp. nov. (*Botryosphaeriales*) from Russia. Mycosphere.

de Wet, J., Wingfield, M.J., Coutinho, T.A., Wingfield, B.D., 2000. Characterization of *Sphaeropsis sapinea* Isolates from South Africa, Mexico, and Indonesia. Plant Disease 84, 151–156.

Delgado-Cerrone, L., Mondino-Hintz, P., Alaniz-Ferro, S., 2016. *Botryosphariaceae* species associated with stem canker, die-back and fruit rot on apple in Uruguay. European Journal of Plant Pathology 1–19

Denman, S., Crous, P.W., Taylor, J.E., Kang, J.C., Pascoe, I., Wingfield, M.J., 2000. An overview of the taxonomic history of *Botryosphaeria*, and a re-evaluation of its anamorphs based on morphology and ITS rDNA phylogeny. Molecules, morphology and classification: towards monophyletic genera in the Ascomycetes. Studies in. Mycology 45, 129–140.

Desprez-Loustau, M.-L., Marçais, B., Nageleisen, L.-M., Piou, D., Vannini, A., 2006. Interactive effects of drought and pathogens in forest trees. Annals of Forest Science 63, 597–612.

Fan, X., Hyde, K.D., Liu, J., Liang, Y., Tian, C., 2015. Multigene phylogeny and morphology reveal *Phaeobotryon rhois* sp. nov. (*Botryosphaeriales*, *Ascomycota*). Phytotaxa 205, 90.

Farr, D.F., Rossman, A.Y., 2016. Fungal Databases, Systematic Mycology and Microbiology Laboratory, ARS, USDA. Accessed August 5, 2016. URL http://nt.ars-grin.gov/fungaldatabases.

Gure, A., Slippers, B., Stenlid, J., 2005. Seed-borne *Botryosphaeria spp*. from native *Prunus* and *Podocarpus* trees in Ethiopia, with a description of the anamorph *Diplodia rosulata* sp. nov. Mycological Research 109, 1005–1014.

Hall, T., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series

Johnson, G.I., Mead, A.J., Cooke, A.W., Dean, J.R., 1992. Mango stem end rot pathogens - Fruit infection by endophytic colonisation of the inflorescence and pedicel. Annals of Applied. Biology 120, 225–234.

Kim, K.W., Park, E.W., Ahn, K.K., 1999. Pre-Penetration Behavior of *Botryosphaeria dothidea* on Apple Fruits. The Plant Pathology Journal 15, 223–227.

Kim, K.W., Park, E.W., Kim, K.S., 2004. Glyoxysomal Nature of Microbodies

Complexed with Lipid Globules in *Botryosphaeria dothidea*. Phytopathology 94, 970–977.

Kim, K.W., Park, E.W., Kim, Y.H., Ahn, K.K., Kim, P.G., Kim, K.S., 2001. Latency- and Defense-Related Ultrastructural Characteristics of Apple Fruit Tissues Infected with *Botryosphaeria dothidea*. Phytopathology 91, 165–72.

Kumar, S., Stecher, G., Tamura, K., 2016. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. Molecular Biology and evolution. msw054.

Li, G.J., Hyde, K.D., Zhao, R.L., Hongsanan, S., Abdel-Aziz, F.A., Abdel-Wahab, M.A., Alvarado, P., Alves-Silva, G., Ammirati, J.F., Ariyawansa, H.A., Baghela, A., Bahkali, A.H., Beug, M., Bhat, D.J., Bojantchev, D., Boonpratuang, T., Bulgakov, T.S., Camporesi, E., Boro20, M.C., Ceska21, O., Chakraborty, D., Chen, J.J., Chethana, K.W.T., Chomnunti, P., Consiglio, G., Cui, B.K., Dai, D.Q., Dai, Y.C., Daranagama, D.A., Das, K., Dayarathne, M.C., Crop, E.D., Oliveira, R.J.V.D., Souza, C.A.F.D., Souza, José I. de; Dentinger, B.T.M., Dissanayake, A.J., Doilom, M., Drechsler-Santos, E.R., Ghobad-Nejhad, M., Gilmore, S.P., Góes-Neto, A., 2016. Fungal diversity notes. Fungal Diversity 75, 27–274.

Linaldeddu, B.T., Deidda, A., Scanu, B., Franceschini, A., Alves, A., Abdollahzadeh, J., Phillips, A.J.L., 2016. Phylogeny, morphology and pathogenicity of *Botryosphaeriaceae*, *Diatrypaceae* and *Gnomoniaceae* associated with branch diseases of hazelnut in Sardinia (Italy). European Journal of Plant Pathology 1–21.

Luchi, N., Ma, R., Capretti, P., Bonello, P., 2005. Systemic induction of traumatic resin ducts and resin flow in Austrian pine by wounding and inoculation with *Sphaeropsis sapinea* and *Diplodia scrobiculata*. Planta 221, 75–84.

Lupo, S., Tiscornia, S., Bettucci, L., 2001. Endophytic fungi from flowers, capsules and seeds of *Eucalyptus globulus*. Revista Iberoamericana de Micología 18, 38–41.

Ma, Z., Morgan, D., Michailides, T., 2001. Effects of water stress on

Botryosphaeria blight of pistachio caused by *Botryosphaeria dothidea*. Plant Disease 85, 745–749.

Maresi, G., Ambrosi, P., Battisti, A., 2002. Pine dieback by *Sphaeropsis sapinea* in Northern and Central Italy. In: Shoot and foliage diseases. Proceedings of the IUFRO Working Party 7.02.02. Finnish Forest Research Institute, Research Papers 829. Hyytiala, Finl. 60–67.

Michailides, T., 1991. Pathogenicity, distribution, sources of inoculum, and infection courts of *Botryosphaeria dothidea* on pistachio. Phytopathology 81, 566–573.

Mohali, S.R., Slippers, B., Wingfield, M.J., 2007. Identification of *Botryosphaeriaceae* from *Eucalyptus* , *Acacia* and *Pinus* in Venezuela. Fungal Diversity 25, 103–125.

Möller, E.M., Bahnweg, G., Sandermann, H., Geiger, H.H., 1992. A simple and efficient protocol for isolation of high molecular weight DNA from filamentous fungi, fruit bodies, and infected plant tissues. Nucleic Acids Research 20, 6115–6116.

O'Donnell, K., 1993. Fusarium and its near relatives. The fungal holomorph: mitotic, meiotic and pleomorphic speciation in fungal systematics, in: Fungal Systematics 225–233.

Paoletti, E., Danti, R., Strati, S., 2001. Pre-and post-inoculation water stress affects *Sphaeropsis sapinea* canker length in *Pinus halepensis* seedlings. Forest Pathology 31, 209–218.

Parker, I., Gilbert, G., 2004. The evolutionary ecology of novel plant-pathogen interactions. Annual Review of Ecology, Evolution, and Systematics 35, 675–700.

Pavlic, D., Slippers, B., Coutinho, T.A., Wingfield, M.J., 2007. *Botryosphaeriaceae* occurring on native *Syzygium cordatum* in South Africa and their potential threat to *Eucalyptus*. Plant Pathology 56, 624–636.

Phillips, A.J.L., Alves, A., Abdollahzadeh, J., Slippers, B., Wingfield, M.J.,

Groenewald, J.Z., Crous, P.W., 2013. The *Botryosphaeriaceae*: genera and species known from culture. Studies in Mycology 76, 51–167.

Phillips, A.J.L., Alves, A., Pennycook, S.R., Johnston, P.R., Ramaley, A., Akulov, A., Crous, P.W., 2008. Resolving the phylogenetic and taxonomic status of dark-spored teleomorph genera in the *Botryosphaeriaceae*. Persoonia - Molecular Phylogeny and Evolution of Fungi 21, 29–55.

Phillips, A.J.L., Lopes, J., Abdollahzadeh, J., Bobev, S., Alves, A., 2012. Resolving the *Diplodia* complex on apple and other *Rosaceae* hosts. Persoonia - Molecular Phylogeny and Evolution of Fungi 29, 29–38.

Phillips, A.J.L., Rumbos, I.C., Alves, A., Correia, A., 2005. Morphology and phylogeny of *Botryosphaeria dothidea* causing fruit rot of olives. Mycopathologia 159, 433–439.

Reay, S., Thwaites, J., Farrell, R., Glare, T., 2006. The lack of persistence of *Ophiostomataceae* fungi in *Pinus radiate* 3 years after damage by the bark beetle *Hylastes ater*, and the subse- quent colonization by *Sphaeropsis sapinea*. Forest Ecology 233, 149–152.

Rodríguez, F., Oliver, J.L., Marín, A., Medina, J.R., 1990. The general stochastic model of nucleotide substitution. Journal of Theoretical Biology 142, 485–501.

Saikkonen, K., Faeth, S.H., Helander, M., Sullivan, T.J., 1998. Fungal endophytes: a continuum of interactions with host plants, in: Annual Review of Ecology and Systematics 319–343.

Sánchez, M.E., Venegas, J., Romero, M.A., Phillips, A.J.L., Trapero, A., 2003. Botryosphaeria and Related Taxa Causing Oak Canker in Southwestern Spain. Plant Disease. 87, 1515–1521.

Schoch, C.L., Shoemaker, R.A., Seifert, K.A., Hambleton, S., Spatafora, J., Crous, P.W., 2006. A multigene phylogeny of the *Dothideomycetes* using four nuclear loci. Mycologia 98, 1041–1052.

Schoeneweiss, D.F., 1981. The Role of Environmental Stress in Diseases of Woody Plants. Plant Disease. 65, 308–314.

Sieber, T.N., 2007. Endophytic fungi in forest trees: are they mutualists? Fungal Biol. Rev. 21, 75–89.

Slippers, B., 2005. Emerging pathogens: fungal host jumps following anthropogenic introduction. Trends in Ecology & Evolution 20, 420–421.

Slippers, B., Boissin, E., Phillips, A.J.L., Groenewald, J.Z., Lombard, L., Wingfield, M.J., Postma, A., Burgess, T., Crous, P.W., 2013. Phylogenetic lineages in the *Botryosphaeriales*: a systematic and evolutionary framework. Studies in Mycology 76, 31–49.

Slippers, B., Crous, P.W., Denman, S., Coutinho, T. a, Wingfield, B.D., Wingfield, M.J., 2004. Combined Multiple Gene Genealogies and Phenotypic Characters Differentiate Several Species Previously Identified as *Botryosphaeria dothidea*. Mycologia 96, 83.

Slippers, B., Wingfield, M.J., 2007. *Botryosphaeriaceae* as endophytes and latent pathogens of woody plants: diversity, ecology and impact. Fungal Biology Reviews 21, 90–106.

Smith, H., Crous, P.W., Wingfield, M.J., Coutinho, T.A., Wingfield, B.D., 2001. *Botryosphaeria eucalyptorum* sp. nov., a New Species in the *B. dothidea*-Complex on Eucalyptus in South Africa. Mycologia 93, 277.

Smith, H., Kemp, G., Wingfield, M., 1994. Canker and die- back of *Eucalyptus* in South Africa caused by *Botryosphaeria dothidea*. Plant Pathology.

Smith, H., Wingfield, M.J., Petrini, O., 1996. *Botryosphaeria dothidea* endophytic in *Eucalyptus grandis* and *Eucalyptus nitens* in South Africa. Forest Ecology and Management 89, 189–195.

Stanosz, G.R., Blodgett, J.T., Smith, D.R., Kruger, E.L., 2002. Water stress and *Sphaeropsis sapinea* as a latent pathogen of red pine seedlings. New Phytologist. 149, 531–538.

Stone, J., Petrini, O., 1997. Endophytes of Forest Trees: a Model for Fungus-Plant Interactions, in: Plant Relationships Part B. Springer Berlin Heidelberg, Berlin, Heidelberg 129–140.

Swart, W.J., Wingfield, M.J., 1991. Biology and control of *Sphaeropsis sapinea* on *Pinus* species in South Africa. Plant Disease 75, 761–766.

Tamura, K., Nei, M., 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Molecular Biology and Evolution 10, 512–26.

Taylor, J.W., Jacobson, D.J., Kroken, S., Kasuga, T., Geiser, D.M., Hibbett, D.S., Fisher, M.C., 2000. Phylogenetic Species Recognition and Species Concepts in Fungi. Fungal Genetics and Biology 31, 21–32.

Thompson, J., 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Research. 25, 4876–4882.

van Niekerk, J.M., Crous, P.W., Groenewald, J.Z. (Ewald), Fourie, P.H., Halleen, F., 2004. DNA phylogeny, morphology and pathogenicity of *Botryosphaeria* species on grapevines. Mycologia 96, 781–798.

White, T., Bruns, T., Lee, S., Taylor, J., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. PCR protocols: a Guide to Methods and Applications 18, 315–322.

Wyka, S.A., Broders, K.D., 2016. The new family Septorioideaceae, within the *Botryosphaeriales* and *Septorioides* strobi as a new species associated with needle defoliation of *Pinus strobus* in the United States. Fungal Biology. 1–11.

Zwolinski, J.B., Swart, W.J., Wingfield, M.J., 1990. Economic impact of a post-hail outbreak of dieback induced by *Sphaeropsis sapinea*. Forest Pathology 20, 405–411.